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Curso de Pós-Graduação em Patologia Humana

TESE DE DOUTORADO

A INFLUÊNCIA DO PRÉ-DIABETES E DIABETES NO AGRAVAMENTO DA COVID-19: UMA ABORDAGEM IMUNOLÓGICA

ICARO BONYEK SANTOS DA SILVA

Salvador – BA

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Tese apresentada ao Curso de Pós-Graduação em Patologia Humana para a obtenção do grau de Doutor**.**

Orientadora: Profa. Dra. Natalia Machado Tavares

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RESUMO

INTRODUÇÃO: Com milhões de casos e mortes confirmadas em todo o mundo, a pandemia da doença do coronavírus 2019 (COVID-19), causada pela infecção do novo coronavírus, denominada síndrome respiratória aguda grave coronavírus 2 (SARS-CoV-2), se tornou uma preocupação mundial. Sabe-se que níveis elevados de glicose na corrente sanguínea são um dos principais fatores de risco para o agravamento da doença. No entanto, ainda não estão elucidados os reais mecanismos envolvidos no agravamento da COVID-19 em indivíduos com pré-diabetes e diabetes. **OBJETIVO**: O objetivo do presente estudo foi avaliar o papel do diabetes e do pré-diabetes no agravamento da COVID-19. **MATERIAL E MÉTODOS**: Inicialmente, foram identificados possíveis genes relacionados a gravidade da COVID-19 em indivíduos com diabetes a partir de dados públicos de expressão gênica em células mononucleares do sangue periférico (PBMCs). Em seguida, foram validados alvos de importância para a infecção do SARS-CoV-2 em PBMCs de pacientes com e sem diabetes. Dosagem de mediadores inflamatórios sistêmicos e análises de correlação foram utilizados para identificar fatores associados à gravidade da COVID-19 em pacientes com a glicemia alterada. **RESULTADOS**: As análises de dados globais públicos revelaram a via do metabolismo do leucotrieno como uma via potencialmente associada a condições respiratórias em pacientes com diabetes. Em seguida, identificamos, na fase aguda da COVID-19, um aumento na expressão dos genes ALOX5 e ACE2/TMPRSS2 em PBMCs de indivíduos com diabetes. Nesses pacientes, também foi encontrado um aumento dos níveis séricos de LTB_4 e IL-6 em comparação a pacientes sem diabetes. O aumento de IL-6 observado em indivíduos com diabetes estava associado com maior internação em unidade de terapia intensiva. Em conjunto, os dados mostram que o diabetes, com a participação da via do LTB_4, pode levar a COVID-19 grave, induzindo lesão pulmonar mais intensa e maior tempo de doença. Curiosamente, pacientes com pré-diabetes, sob a participação da produção de IL-6, também evoluem para a COVID-19 grave em maior frequência, considerando a redução nas taxas de troca gasosa e maior tempo de hospitalização. Contudo, o pré-diabetes não induziu sequelas da COVID-19 distintas daquelas de indivíduos sem diabetes. Além disso, nós mostramos também, que exames laboratoriais de rotina podem ser utilizados para identificar altos/baixos produtores de IL-6, cujos níveis estão relacionados com a gravidade da COVID-19. **CONCLUSÃO**: O aumento da produção de LTB_4 e IL-6 observado em indivíduos com diabetes e pré-diabetes, respectivamente, pode piorar o desfecho da COVID-19.

Palavras-chave: COVID-19. Diabetes. Inflamação. IL-6. LTB_4. Pré-diabetes.

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ABSTRACT

INTRODUCTION: With millions of confirmed cases and deaths worldwide, the coronavirus disease 2019 (COVID-19) pandemic caused by the infection of the novel coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a worldwide concern. It is known that high levels of glucose in the bloodstream is one of the main risk factors for the worsening of the disease. However, the real mechanisms involved in the aggravation of COVID-19 in individuals with prediabetes and diabetes are not yet elucidated. **OBJECTIVE**: Thus, the aim of the present study was to evaluate the influence of diabetes and pre-diabetes on the worsening of COVID-19. **MATERIAL AND METHODS**: Initially, possible genes related to the severity of COVID-19 in individuals with diabetes were identified from public gene expression data in peripheral blood mononuclear cells (PBMCs). Then, we validated important targets for SARS-CoV-2 infection in PBMCs of patients with and without diabetes. Systemic inflammatory mediators and correlation analyzes were performed to identify factors associated with COVID-19 severity in patients with altered blood glucose. **RESULTS**: Public data analyzes revealed the leukotriene metabolism as a pathway potentially associated with respiratory conditions in patients with diabetes. Thus, we identified, during the acute phase of COVID-19, an increase in the expression of ALOX5 and ACE2/TMPRSS2 in PBMCs of individuals with diabetes. In these patients, we also found an increase in serum levels of LTB_4 and IL-6 compared to patients without diabetes. The increase in IL-6 observed in individuals with diabetes was associated with longer intensive care unit admission. Taken together, the data show that diabetes, with the participation of the LTB_4 pathway, induces a more severe disease, with more intense lung damage (gas exchange rates) and longer disease duration. Interestingly, patients with prediabetes, under the participation of IL-6 production, also develop to severe COVID-19 more frequently, considering the reduction in gas exchange rates and longer hospitalization time. However, prediabetes did not induce sequelae of COVID-19 distinct from those of subjects without diabetes. In addition, we also show that routine laboratory tests can be used to identify different producers of IL-6, which is related to the severity of COVID-19. **CONCLUSION**: The increased production of LTB4 and IL-6 seen in individuals with diabetes and prediabetes, respectively, may worsen the outcome of COVID-19.

Keywords: COVID-19. Diabetes. Inflammation. IL-6. LTB_4. Prediabetes.

LISTA DE ABREVIATURAS

AA	Ácido Araquidônico
AGE	Produtos Finais da Glicação Avançada
ALOX5	Gene codificador da enzima 5-lipoxigenase
ALT	Alanina Aminotransferase
AST	Aspartato aminotransferase
BLT1	Receptor de Leucotrieno B4 do tipo 1
BLT2	Receptor de Leucotrieno B4 do tipo 2
CK	Creatinofosfoquinase
COVID-19	Doença do Coronavírus 2019
CoVs	Coronavírus
DM1	Diabetes do tipo 1
DM2	Diabetes do tipo 2
DPOC	Doença Pulmonar Obstrutiva Crônica
DPP4	Enzima Dipeptidil Peptidase 4
E	Proteína do Envelope
ECA2	Enzima Conversa da Angiotensina 2
ERO	Espécies Reativas de Oxigênio
EUA	Estados Unidos da América
FLAP	Proteína Ativadora da 5-Lipoxigenase
FiO_2	Fração inspirada de Oxigênio
G-CSF	Fator Estimulador de Colônias de Granulócitos

GLP-1	Peptídeo Semelhante a Glucagon 1
GLUT4	Transportador de Glicose 4
HbA1c	Hemoglobina glicada
HCoVs	Coronavírus humanos
IFN	Interferons
IL	Interleucina
LDH	Lactato Desidrogenase
LTB_4	Leucotrieno B4
M	Proteína de Membrana
MCP1	Proteína 1 Quimioatraente de Monócitos
MERS-CoV	Coronavírus da Síndrome Respiratória do Oriente Médio
MIP1α	Proteína Inflamatória Macrofágica 1 alfa
N	Proteína do Nucleocapsídeo
NDM	Sem diabetes
NLR	Receptores do tipo NOD
OMS	Organização Mundial da Saúde
NO	Óxido Nítrico
PAMPS	Padrões Moleculares Associados a Patógenos
PaO_2	Pressão parcial de Oxigênio dissolvida no sangue arterial
PASC	Sequelas pós-aguda de infecção por SARS-CoV-2
PBMCs	Células Mononucleares do Sangue Periférico
PCR	Proteína C Reativa
PDM	Pré-diabetes

PKC	Proteína Quinase C
RBD	Domínio de ligação ao receptor
RI	Resistência a Insulina
RIG-I	gene I induzível por ácido retinóico
RNA	Ácido Ribonucleico
S	Proteína Spike
SARS	Síndrome Respiratória Aguda Grave
SARS-CoV	Coronavírus da Síndrome Respiratória Aguda Grave
SARS-CoV-2	Novo Coronavírus da Síndrome Respiratória Aguda Grave
SBD	Sociedade Brasileira de Diabetes
SDRA	Síndrome do Desconforto Respiratório Agudo
SpO_2	Saturação de periférica de Oxigênio
TMPRSS2	Serina Protease Transmembranar 2
TNF	Fator de Necrose Tumoral
TOTG	Teste Oral de Tolerância a Glicose
UTI	Unidade de Terapia Intensiva
VEGF	Fator de Crescimento Vascular Endotelial
VMI	Ventilação Mecânica Invasiva

SUMÁRIO

1 **INTRODUÇÃO**

1.1. DO SARS-CoV À COVID-19

1.1.1 **SARS-CoV, MERS-CoV e o SARS-CoV 2**

Os coronavírus humanos (HCoVs) são membros da família dos coronavírus (CoVs) que são responsáveis por várias doenças respiratórias de gravidade variável como resfriado comum, bronquiolite e pneumonia. Os coronavírus são caracterizados pela presença de uma proteína em formato de espícula, a proteína spike (S), encontrada por toda a superfície do vírus, o que lhe dá um aspecto semelhante a uma coroa (ABDELRAHMAN; LI; WANG, 2020). Além da proteína S, os HCoVs são compostos pelas proteínas do envelope (E), de membrana (M) e do nucleocapsídeo (N).

Dentre os vírus de RNA, os coronavírus possuem o maior genoma (26,4–31,7 kb) e podem ser classificados em quatro gêneros, o alfa, beta, gama e delta (KIRTIPAL; BHARADWAJ; KANG, 2020; WOO et al., 2009). O novo coronavírus 2 da Síndrome Respiratória Aguda Grave (SARS-CoV-2), o vírus da Síndrome Respiratória Aguda Grave (SARS-CoV) e o da Síndrome Respiratória do Oriente Médio (MERS-CoV) pertencem ao grupo dos *betacoronavírus* e estão entre os sete coronavírus capazes de infectar humanos (ABDELRAHMAN; LI; WANG, 2020). Os demais coronavírus causam um quadro de resfriado comum e apresentam ampla distribuição mundial.

Com casos confirmados em 28 países ao redor do Mundo e deixando 774 mortos (taxa de letalidade de 10-15%), o SARS-CoV foi o primeiro membro dos coronavírus a induzir uma pandemia com início em 2002, na China (ABDELRAHMAN; LI; WANG, 2020; KIRTIPAL; BHARADWAJ; KANG, 2020). O SARS-CoV tem como possíveis reservatórios naturais os morcegos e como reservatório intermediário os gatos civetas. Sua transmissão se dá por meio de aerossóis, gotículas e contato (ABDELRAHMAN; LI; WANG, 2020; HU et al., 2017). Durante a interação parasito-hospedeiro o seu principal ligante é a enzima conversora da angiotensina 2 (ECA2) por meio do domínio de ligação ao receptor (RBD) da proteína S (LI et al., 2005). Com uma incubação de 2-7 dias, a leucopenia, linfopenia e trombocitopenia são as principais características hematológicas encontradas em pacientes infectados pelo SARS-CoV (ABDELRAHMAN; LI; WANG, 2020).

Uma década depois da Síndrome Respiratória Aguda Grave (SARS) provocada pelo SARS-CoV, tendo como reservatório intermediário, camelos dromedários, o MERS-CoV

emergiu em países do Oriente Médio. Com diagnóstico confirmado em 27 países, a MERS resultou em 858 casos fatais. Possuindo uma taxa de letalidade maior que a do SARS-CoV, com cerca de 35%, a interação com as células do hospedeiro difere da cepa da pandemia anterior, sendo mediada, pela interação da porção RBD da proteína S com a enzima dipeptidil peptidase 4 (DPP4), uma proteína encontrada na superfície celular (ABDELRAHMAN; LI; WANG, 2020; CUI; LI; SHI, 2019; KIRTIPAL; BHARADWAJ; KANG, 2020). Com transmissão semelhante ao SARS-CoV, após a infecção, o MERS-CoV tende a permanecer em incubação por um período maior, em torno de 2-14 dias, causando leucocitose e monocitose em pacientes acometidos pela doença (ABDELRAHMAN; LI; WANG, 2020; PARK et al., 2017).

Um terceiro episódio epidemiológico ocorreu envolvendo os HCoVs, em dezembro de 2019 na província de Wuhan, na China. Uma nova cepa homóloga ao SARS-CoV, responsável pela Doença do Coronavírus 2019 (COVID-19), foi nomeada como SARS-CoV-2. Análises genômicas mostraram, com 96% de similaridade a outros coronavírus nativos, que o SARS-CoV-2 tem os morcegos como primeiro hospedeiro e com 99% os pangolins (*Manis spp.*) como hospedeiro intermediário. No entanto, outros estudos ainda estão sendo realizados para melhor compreender a origem e evolução do SARS-CoV-2 (KIRTIPAL; BHARADWAJ; KANG, 2020; YI et al., 2020; ZHOU et al., 2020b). O modo de transmissão do SARS-CoV-2 se dá como os demais HCoVs (SARS e MERS-CoV) e o modo de interação com a célula hospedeira se assemelha ao SARS-CoV, a partir da interação entre a proteína S do vírus com a ECA2 da célula hospedeira. Com um período de incubação de 2-14 dias, o SARS-CoV-2 possui uma taxa de letalidade menor que os outros HCoVs, cerca de 1-5%, no entanto, possui uma alta taxa de transmissibilidade, o que resulta em um grande número de infectados e de óbitos em todo o Mundo (ABDELRAHMAN; LI; WANG, 2020; LI et al., 2021). Com um número muito superior de indivíduos acometidos pela COVID-19, a infecção pelo SARS-CoV-2 foi reportada em 216 países espalhados em todos os seis continentes (HU et al., 2021).

1.1.2 **A COVID-19**

Segundo o painel de controle da Organização Mundial da Saúde (OMS), a pandemia da COVID-19 se tornou a enfermidade provocada por HCoVs mais devastadora até o momento (WHO, 2021). Até 09 agosto de 2022, segundo a OMS, os casos diagnosticados com a COVID-19 já ultrapassam o número de 500 milhões em todo o Mundo, sendo que 6 milhões destes, foram a óbito.

O primeiro caso da doença foi reportado em 8 de dezembro de 2019 em Wuhan, na China. Em 31 de Dezembro de 2019 a secretaria de saúde de Wuhan informou publicamente a OMS sobre o surto de pneumonia com causa ainda indefinida, apresentando sintomas de pneumonia viral, incluindo febre, tosse e desconforto torácico e, em casos graves, dispneia e infiltração pulmonar bilateral. Em 09 de Janeiro de 2020 foi sequenciado e anunciado o agente etiológico do novo surto e, meses depois, em 11 de março de 2020, a COVID-19 provocada pelo SARS-CoV-2, foi definida como uma pandemia pela OMS (HU et al., 2021; ZHU et al., 2020).

1.1.3 **A imunopatogênese**

Apesar de estruturalmente os HCoVs serem simples, a patogênese da COVID-19 é complexa e não totalmente compreendida, tendo início a partir da interação entre proteínas patógeno-hospedeiro, resultando na liberação de uma tempestade de mediadores inflamatórios e dano tecidual exacerbado (TAY et al., 2020).

A infecção pelo SARS-CoV-2 inicia-se a partir da ligação do domínio de ligação ao receptor (RBD, subunidade 1) da proteína S do vírus com a enzima conversora de angiotensina 2 (ECA2), presente em células epiteliais da via aérea, células epiteliais alveolares, células endoteliais e macrófagos residentes no pulmão (JIA et al., 2005; TAY et al., 2020). Apesar da ECA2 ser o ligante com maior afinidade, outro receptor também tem sido estudado como alvo de ligação do SARS-CoV-2, como CD147, também conhecido como Basigin ou EMMPRIN. No entanto, estudos com ECA2 tem sido mais frequentes, tornando essa via mais amplamente explorada e elucidada (ING et al., 2020). A ligação entre a RBD do vírus e a ECA2 da célula hospedeira, juntamente com a subunidade 2 (S2) da proteína S, ativa o processo de endocitose e fusão membranar, respectivamente (KIRTIPAL; BHARADWAJ; KANG, 2020; TAY et al., 2020). Além da interação entre RBD e ECA2, proteases como a serina transmembranar 2 (TMPRSS2), furin e catepsina L são necessárias para permitir a entrada do vírus no citoplasma da célula hospedeira (HU et al., 2021).

O processo de internalização, replicação e liberação viral não é silencioso do ponto de vista imunológico, devido a ativação de diversos receptores de reconhecimento de padrão, como os receptores do tipo Toll, do tipo NOD (NLR) e gene I induzível por ácido retinóico (RIG-I). Além disso, há indução de diferentes vias de morte celular, como apoptose, piroptose e necrose das células infectadas (ABDELRAHMAN; LI; WANG, 2020; KIRTIPAL; BHARADWAJ;

KANG, 2020; MUNIYAPPA; GUBBI, 2020; TAY et al., 2020). O reconhecimento e a indução de morte celular resultam no recrutamento de células imune como monócitos, macrófagos e posteriormente células T e B. Essa quimiotaxia é acompanhada pela liberação de mediadores inflamatórios como a interleucina (IL) do tipo 6, IL-1b, IL-2, IL-7, IL-10, interferon-gama (IFN-γ), proteína 10 induzida por interferon gama (IP-10), fator estimulador de colônias de granulócitos (G-CSF), proteína 1 quimioatraente de monócitos (MCP1), proteína inflamatória macrofágica 1 alfa (MIP1α), fator de necrose tumoral (TNF) e Leucotrieno B_4 (LTB_4) (BONYEK-SILVA et al., 2021; HAN et al., 2020; HUANG et al., 2020; PÉREZ et al., 2021; TAY et al., 2020). A liberação exacerbada desses mediadores, conhecida como tempestade de citocinas, está relacionada com a forma grave da doença e presente em maior frequência em pacientes não sobreviventes (HAN et al., 2020; TAY et al., 2020; ZHOU et al., 2020a, 2020c).

Dentre os mediadores inflamatórios, a citocina IL-6 é um dos principais na tempestade de citocinas decorrente da COVID-19. Associado com a gravidade da doença, a IL-6 possui funções pleiotrópicas a depender do seu local e receptor ligante. Na COVID-19, alternativas terapêuticas buscam a inibição da ação da IL-6 como forma de atenuar a doença, sendo que um dos medicamentos mais utilizados nesse contexto é o Tocilizumabe, um anticorpo monoclonal inibidor competitivo da sinalização mediada por IL-6 (IL-6R) (GUBERNATOROVA et al., 2020; ROSE-JOHN; WINTHROP; CALABRESE, 2017).

A tempestade de citocinas, o infiltrado inflamatório, a liberação de espécies reativas de oxigênio (EROS) e o dano direto provocado pelo vírus a célula hospedeira, em conjunto, causam dano tecidual pulmonar caracterizado por descamação de células alveolares, formação de membrana hialina e edema pulmonar (TAY et al., 2020; XU et al., 2020). Essas alterações no tecido pulmonar resultam em uma ineficiência nas trocas gasosas e uma menor saturação de oxigênio, comprometendo assim a manutenção dos tecidos, o que dificulta a recuperação do paciente ou leva ao óbito por insuficiência respiratória (TAY et al., 2020).

1.1.4 **Manifestações clínicas**

O SARS-CoV-2 tem uma capacidade de transmissão muito alta, fazendo com que um número expressivo de pessoas seja infectado em um intervalo curto de tempo. Isso faz com que o vírus encontre diferentes perfis de hospedeiro, seja pela etnia, idade, sexo ou até mesmo por comorbidades preexistentes. Nesse contexto, a COVID-19 apresenta diferentes formas clínicas, podendo ser classificada como assintomática, leve, moderada e grave (BRODIN, 2021). Os

assintomáticos apesar de possuírem teste molecular positivo, não apresentam nenhum sintoma clínico aparente. Os indivíduos com a forma leve da doença apresentam poucos sintomas como febre, dor de garganta, tosse seca, mal-estar, dores no corpo, náuseas, vômitos, dor abdominal e diarreia. Nessa forma clínica dificilmente os indivíduos necessitam de suporte hospitalar. Por outro lado, na forma moderada, os pacientes apresentam sintomas mais característicos de pneumonia, como tosse e febre persistente. Nesses casos, é necessário a internação em leitos clínicos, porém sem a necessidade de ventilação mecânica. Entretanto, na forma grave, o paciente apresenta desconforto respiratório, possuindo uma saturação (SpO_2) menor ou igual a 93% e pressão parcial de oxigênio (PaO_2) em razão da concentração de oxigênio inspirada (FiO_2) no sangue arterial (PaO_2/FiO_2) menor ou igual a 300 mmHg. Além desses parâmetros, acima de 50% do pulmão apresenta comprometimento funcional a partir de exames de imagem. Na forma grave, os pacientes necessitam de internação em Unidade de Terapia Intensiva (UTI) e frequentemente o uso de ventilação mecânica invasiva (VMI) na tentativa de reverter esse quadro ou minimizar o dano pulmonar (BRODIN, 2021; HAN et al., 2020; PARASHER, 2021).

De acordo com um estudo realizado na China com 72.314 casos de COVID-19, a maioria dos pacientes desenvolvem a forma leve da doença (81%), a forma grave afeta 14% dos casos e 5% são enquadrados clinicamente como críticos (pacientes que apresentavam insuficiência respiratória, choque séptico e/ou disfunção ou insuficiência de múltiplos órgãos) (HU et al., 2021; WU; MCGOOGAN, 2020).

1.1.5 **Sintomas, sinais e características laboratoriais**

A COVID-19 é marcada por uma variedade de sinais e sintomas que dependem da gravidade da doença. Os sintomas mais frequentes são febres, fadiga, tosse, náusea, dor de cabeça, diarreia e produção de escaro. Já os sinais mais relatados são a congestão na garganta, inchaço de amígdala e aumento dos gânglios linfáticos (GUAN et al., 2020; LI et al., 2021).

Durante a fase aguda da doença, além do aumento na produção de diversos mediadores inflamatórios alguns parâmetros laboratoriais sanguíneos também são alterados e utilizados como biomarcadores da gravidade da doença, como proteína C reativa (PCR), dímero-D, alanina aminotransferase (ALT), aspartato aminotransferase (AST), ureia, creatinofosfoquinase (CK), creatinina, lactado desidrogenase (LDH) e procalcitonina. Os achados hematológicos mais comuns são a linfocitopenia, trombocitopenia e neutrofilia, sendo essas alterações mais predominantes em pacientes com maior gravidade da doença (GUAN et al., 2020; LI et al.,

2021). Achados em nível radiológico e gasométrico, como área em vidro fosco, diminuição de SpO_2 e PaO_2/FiO_2, respectivamente, são encontrados principalmente em pacientes com COVID-19 grave (GUAN et al., 2020; MUDATSIR et al., 2020; PARASHER, 2021).

1.1.6 **Sequelas**

Alguns termos são utilizados para definir as sequelas que a COVID-19 pode provocar, tais como: sequela pós-aguda de infecção por SARS-CoV-2 (PASC), Síndrome Pós-COVID, COVID-19 longa ou COVID-19 de longo prazo (LOPEZ-LEON et al., 2021). Estes sintomas podem persistir por duas ou mais semanas após a fase aguda.

Na COVID-19 de longo prazo foram encontrados diversos sintomas residuais, sendo os mais frequentes fadiga, dor de cabeça, déficit de atenção, perda de cabelo e falta de ar (LOPEZ-LEON et al., 2021). Em até 6 meses após a fase aguda alguns outros sintomas tem sido relatados, como fraqueza muscular, dificuldade para dormir, ansiedade e depressão (HUANG et al., 2021). Apesar de já serem descritos, ainda não estão estabelecidos quais os reais fatores de risco para a persistência desses sintomas. Alguns estudos têm demostrado que o sexo feminino e a gravidade da doença podem ter influência nestas sequelas. No entanto, ainda é necessário considerar que essa associação ao sexo seja um viés sociocultural e não necessariamente devido a doença, uma vez que mulheres tendem a ser mais cuidadosas e atentas à saúde, além de buscarem atendimento médico com mais frequência (DENNIS et al., 2021; HUANG et al., 2021; LOPEZ-LEON et al., 2021). Além dos sintomas descritos acima, danos em múltiplos órgãos tem sido identificados, como dano cardíaco, pulmonar, renal, hepático, pancreático e em órgãos linfoides secundários como o baço (DENNIS et al., 2021). Isso reflete na persistência de alterações em alguns marcadores laboratoriais como PCR, ferritina, dímero-D, procalcitonina e IL-6 (LOPEZ-LEON et al., 2021).

Ademais, o diagnóstico de diabetes tem sido reportado na COVID-19 de longo prazo e denominado de diabetes recente associado a COVID-19. É caracterizado por indivíduos com hiperglicemia e diagnóstico confirmado para COVID-19 sem histórico de diabetes e com níveis anteriores normais de hemoglobina glicada (HbA1c). No entanto, ainda são desconhecidos o tipo específico do diabetes que acomete esses pacientes, bem como o mecanismo envolvido nesta complicação (LOPEZ-LEON et al., 2021; RUBINO et al., 2020).

1.1.7 **Fatores de risco**

Desde o início da pandemia de COVID-19, médicos e cientistas têm observado que um grupo de indivíduos tem desenvolvido a forma moderada/grave da doença com maior frequência. Logo nos primeiros estudos publicados foram identificadas características em comum entre esses indivíduos, o que definiu os fatores de risco para o desenvolvimento da COVID-19 grave. Dentre os principais fatores de risco descritos então: idade, sexo, pacientes imunocomprometidos e a presença de comorbidades, tais como obesidade, hipertensão, doença pulmonar obstrutiva crônica (DPOC) e o diabetes (BRODIN, 2021; MUDATSIR et al., 2020; RICHARDSON et al., 2020).

A idade avançada em indivíduos infectados pelo SARS-CoV-2 mostrou ser um fator importante para o agravamento da doença em comparação a pessoas mais jovens (GUAN et al., 2020). Além disso, indivíduos do sexo masculino parecem ter maior predisposição para o agravamento da COVID-19, uma vez que o número de pacientes homens admitidos nas assistências hospitalares com a forma moderada/grave da doença é maior (GUAN et al., 2020; RICHARDSON et al., 2020). Pacientes com câncer e outras doenças sob a utilização de terapias imunossupressoras também são considerados fatores de risco para evolução da forma grave da COVID-19 (BRODIN, 2021; GAO et al., 2020).

Obesidade, hipertensão e diabetes, são doenças destacadas quando se trata de COVID-19 grave (MUDATSIR et al., 2020; RICHARDSON et al., 2020). Essas doenças metabólicas preocupam os médicos e as autoridades públicas de saúde no enfrentamento da pandemia da COVID-19 devido à alta prevalência dessas doenças na população mundial. A prevalência e incidência dessas doenças somadas a alta capacidade de transmissão do SARS-CoV-2, tornam necessários estudos que explorem e busquem alternativas terapêuticas para mitigar o impacto da COVID-19 nessa população.

1.2 DIABETES E COVID-19

1.2.1 **Diabetes em números**

Caracterizado por níveis elevados de glicose na circulação sanguínea, o *Diabetes mellitus* é uma das desordens metabólicas mais frequentes no Mundo (KEANE et al., 2017). Até o período de 2019, segundo a Federação Internacional de Diabetes, 463 milhões de pessoas

na faixa etária de 20-79 anos vivem com diabetes em todo o Mundo, o que corresponde a 9,3% da população (FEDERATION, 2019). De modo preocupante estima-se um aumento gradativo anualmente, podendo chegar a 578 milhões de pessoas (10,2% da população) até o ano de 2030 (FEDERATION, 2019). O aumento dessa prevalência está associado a múltiplos fatores, como a urbanização, transição epidemiológica, mudança nutricional, sedentarismo, obesidade, crescimento, envelhecimento populacional e à maior sobrevida dos indivíduos portadores do diabetes (LYRA et al., 2020).

A lista dos 10 países com mais adultos portadores da doença é liderada pela China (116 milhões de pessoas com diabetes), Índia (77 milhões) e os Estados Unidos da América (EUA) (31 milhões). Com 16,8 milhões de indivíduos com diabetes (8% da população), o Brasil ocupa a 5º posição da lista (FEDERATION, 2019; LYRA et al., 2020). No entanto, 46% da população brasileira apesar de serem portadores da doença ainda desconhecem o seu diagnóstico (FEDERATION, 2019). Neste cenário, há uma maior prevalência de pessoas do sexo masculino comparado com feminino e que vivem em regiões urbanas.

Globalmente, 11,3% das mortes em adultos de 20-79 anos estão atreladas ao diabetes e suas complicações, o que corresponde a 4,2 milhões de pessoas só no ano de 2019 (FEDERATION, 2019). Com uma despesa anual de US$ 52,3 bilhões o Brasil é o terceiro país que mais gasta no mundo devido ao diabetes e suas complicações, ficando atrás apenas do EUA (US$ 249,6 bilhões) e a China (US$ 109 bilhões), os quais lideram a lista (FEDERATION, 2019). Diante de todos esses números, o diabetes tem ganhado grande atenção e se tornado uma preocupação para a saúde pública a nível mundial.

1.2.2 **O que é diabetes**?

A elevação dos níveis de glicose na corrente sanguínea de forma persistente é a principal característica do diabetes e pode ser consequência da não produção do hormônio insulina pelas células b pancreáticas, denominado Diabetes do tipo 1 (DM1) ou pelo reconhecimento ineficiente desse hormônio, levando a um quadro de resistência periférica a insulina, o que configura o Diabetes do tipo 2 (DM2) (KATSAROU et al., 2017; KEANE et al., 2017).

Com uma prevalência de 5 a 10% de todos os casos de diabetes, o DM1 ou insulínico dependente é uma doença autoimune provocada pela reação de autoanticorpos contra antígenos presentes nas células b da ilhota pancreática, as quais são responsáveis pela produção de insulina. Com sintomas como poliúria, polidipsia, fome constante, perda de peso,

comprometimento da visão e fadiga, o tratamento do DM1 é baseado na administração de insulina exógena com a finalidade de normalizar os níveis de glicose da corrente sanguínea (FEDERATION, 2019; LYRA et al., 2020).

Por outro lado, o DM2 é a forma mais frequente da doença, com 85-95% dos casos, e é caracterizada pela resistência a insulina (RI) principalmente nos tecidos periféricos, como o tecido esquelético e adiposo (KEANE et al., 2017; ZHOU et al., 2016). Com sintomatologia semelhante ao DM1, o tratamento do DM2 se dá através de medicamentos normoglicemiantes, como Metformina, Gliclazida, análogos do peptídeo semelhante a glucagon 1 (GLP-1) e os inibidores de DPP4 (IDF -ATLAS DO DIABETES, 2015).

1.2.3 **Normoglicemia, pré-diabetes e diabetes**

Independentemente do tipo de diabetes, a variação glicêmica é um dos mais antigos e principais parâmetros laboratoriais utilizados pela população e sistemas de saúde. A dosagem da concentração dos níveis de glicose na corrente sanguínea consegue confirmar e predizer o diagnóstico do diabetes (FEDERATION, 2019). A glicemia em jejum, o Teste Oral de Tolerância a Glicose (TOTG), glicemia ao acaso e a HbA1c são os testes mais utilizados atualmente em nível mundial para monitoramento e diagnóstico do diabetes. No Brasil, a Sociedade Brasileira de Diabetes (SBD) considera, com base no teste de glicemia em jejum, níveis de glicose menores do que 100 mg/dL, indivíduos normoglicêmicos. Valores maiores ou igual a 100 e menores de 126 mg/dL, indivíduos com risco aumentado para o diabetes, ou simplesmente pré-diabético. Por fim, para aqueles indivíduos que possuem níveis maiores ou igual a 126 mg/dL, o diagnóstico de diabetes estabelecido é confirmado. Com a vantagem de refletir os valores glicêmicos dos últimos 3 e 4 meses e sofrer menos variabilidade dia a dia, o teste de HbA1c é um dos mais robustos para confirmação do diabetes. Nesse tipo de teste, valores menores de 5,7% classifica os indivíduos como normoglicêmicos. Valores acima ou igual a 5,7 e menores do que 6,5% classificam indivíduos com pré-diabetes e valores maiores ou igual a 6,5%, o diagnóstico do diabetes é confirmado. Para aqueles que possuírem valores acima de 200 mg/dL no TOTG e através da glicemia ao acaso também são considerados indivíduos portadores do diabetes (LYRA et al., 2020).

1.2.4 **Complicações associadas ao Diabetes**

O Diabetes é uma doença de caráter crônico e silencioso que, ao longo do tempo, se não tratada pode levar diversas complicações, resultando na redução da qualidade de vida desses pacientes e um aumento dos custos nos sistemas públicos de saúde (FEDERATION, 2019). A elevação da concentração da glicose nos grandes e pequenos vasos do nosso corpo, de forma persistente, pode levar a doenças cardiovasculares, retinopatia, nefropatia, neuropatia, dificuldade de cicatrização, inflamação crônica de baixo grau, suscetibilidade a doenças infeciosas e agravamento de doenças respiratórias (BONYEK-SILVA et al., 2020; BOWLING; RASHID; BOULTON, 2015; BRANDT; SEREZANI, 2017; EZZATI, 2014; FEDERATION, 2019; FILGUEIRAS et al., 2015a; GOLDEN et al., 2015; KATSAROU et al., 2017; KHATEEB; FUCHS; KHAMAISI, 2019; VINCENT et al., 2011; WU et al., 2016).

As doenças cardiovasculares são as causas mais comuns de morte em pacientes com diabetes, dentre estas estão a angina, infarto do miocárdio, acidente vascular cerebral, doença arterial periférica e insuficiência cardíaca (FEDERATION, 2019; SR et al., 2013). As doenças cardiovasculares e diabetes, juntos, em 2010 foram responsáveis por mais de 33% de todas as mortes do mundo (EZZATI, 2014). O paciente com diabetes, principalmente do tipo 2, possui os maiores fatores de risco para as doenças cardiovasculares, como a obesidade, hipertensão, dislipidemia, coagulabilidade, disfunção endotelial e neuropatia (LEON, 2015). A inflamação exacerbada induzida pelo diabetes, com a participação de Interleucina 1 (IL-1), IL-6 e proteína quimioatrante de monócitos (MCP-1) tem sido correlacionado com a disfunção endotelial, infarto do miocárdio e cardiomiopatias (MATHEUS et al., 2013).

A retinopatia diabética, com uma prevalência de 80% em pacientes com DM1, é uma complicação microvascular que afeta de forma moderada ou grave a visão em pacientes portadores do diabetes, podendo progredir até para a perda total da visão (HANG et al., 2014; KATSAROU et al., 2017; WORLD HEALTH ORGANIZATION, 2016). A retinopatia induzida pelo diabetes é caracterizada principalmente pelo extravasamento vascular e a neovascularização, esses dois fatores são provenientes da ativação dos produtos finais da glicação avançada (AGE), ativação da Proteína Quinase C (PKC) e a ativação da via de superóxido. A ativação dessas vias resulta na regulação positiva de fatores imunológicos, imunogênicos e pró-angiogênicos, como fator de crescimento vascular endotelial (VEGF), TNF-α, IL-1, IL-6, IL-8 e MCP-1 (HANG et al., 2014).

O dano induzido pelo diabetes à pequenos vasos sanguíneos localizados nos rins pode levar ao quadro de nefropatia, onde os rins se tornam menos eficientes ou até mesmo totalmente falhos (IDF, 2015; NAVARRO-GONZÁLEZ et al., 2011). O Estagio Final de Doença Renal (EFDR) tem uma incidência de até 10 vezes maior em pacientes com diabetes comparado com aqueles que não são portadores da doença (WORLD HEALTH ORGANIZATION, 2016). A hiperglicemia age de forma direta nas células renais residentes ou não, levando a produção de mediadores inflamatórios e fatores de crescimento, os quais provocam alterações no glomérulo desses pacientes, incluindo arteriosclerose hialina, aumento da deposição de colágeno e aumento da permeabilidade glomerular (SCHENA, 2005).

A Neuropatia diabética, complicação que afeta os nervos, é a complicação mais comum do diabetes, afetando cerca de 50% durante o curso da doença. A participação da hiperglicemia no contexto da neuropatia se dá principalmente pela disfunção mitocondrial ocasionado pela mudança metabólica e consequente produção de espécies reativas de oxigênio (ERO) (VINCENT et al., 2011). A forma mais comum é a neuropatia periférica, a qual os nervos sensoriais dos pés são os mais afetados, podendo levar a dor, formigamento e perda de sensibilidade (BOWLING; RASHID; BOULTON, 2015; IDF -ATLAS DO DIABETES, 2015).

1.2.5 **Inflamação**

A resposta inflamatória, de modo controlado e auto limitante, é um processo biológico de importância fundamental na defesa contra patógenos e para a homeostase dos tecidos (CHEN; NUÑEZ, 2010). No entanto, além dos Padrões Moleculares Associados a Patógenos (PAMPs) que ativam a resposta imunológica, ela pode ser acionada mesmo na ausência desses gatilhos, induzindo uma resposta inflamatória em condições estéreis, denominada de "inflamação estéril". Esse perfil inflamatório é desencadeado pelo acúmulo de produtos metabólicos como colesterol, ácidos graxos livres, ácido úrico ou glicose (BRANDT; SEREZANI, 2017; FILGUEIRAS et al., 2015b).

A alta concentração de glicose no sangue encontrada em indivíduos portadores do diabetes resulta na ativação da inflamação estéril, caracterizada por elevados níveis de mediadores pró-inflamatórios como IL-1b, IL-6, TNF-a e Leucotrieno B_4 (LTB_4) (BALTZIS; ELEFTHERIADOU; VEVES, 2014; FILGUEIRAS et al., 2015b; ZAND; MORSHEDZADEH; NAGHASHIAN, 2017). Esses mediadores estão relacionados com a

resistência à insulina, nos casos de DM2, e no agravamento de outras complicações conhecidamente associadas ao diabetes (BONYEK-SILVA et al., 2020; BORST, 2004; BRANDT et al., 2018; LI et al., 2015; SHI et al., 2019).

Dentre os mediadores proteicos da imunidade inata que participam da imunopatogênese do diabetes e suas complicações, as citocinas IL-1b, IL-6 e TNF-a são as bem mais estudadas. A produção aumentada de IL-1b em ambientes hiperglicêmicos está intimamente ligada com a perda de função, induzida por apoptose, das células beta pancreáticas a partir da ativação do receptor FAS. Além disso, a presença de IL-1b e ácidos graxos livres, juntos, mostrou-se ter um maior impacto na manutenção da inflamação em contextos hiperglicêmicos (DONATH; SHOELSON, 2011). A IL-6 e o TNF-a possuem um papel importante na base do DM2, a resistência à insulina, principalmente em pacientes obesos onde a produção desses mediadores são maiores devido a produção por adipócitos (DONATH; SHOELSON, 2011; WELLEN; HOTAMISLIGIL, 2005). A sinalização comprometida do transportador de glicose do tipo 4 (GLUT4) através da ação do TNF-a é um dos principais mecanismos para resistência à insulina periférica (GREGOR; HOTAMISLIGIL, 2011). O LTB_4 é um mediador lipídico inflamatório derivado da cascata de sinalização do ácido araquidônico (AA) sob a ação da enzima 5-lipoxigenase (5-LO) juntamente com a proteína de ativação (FLAP), o qual após a ação de uma hidrolase, o LTB_4 então formado, pode se ligar ao Receptor de LT tipo 1 (BLT1) ou 2 (BLT2), sendo o BLT1 o de maior afinidade com o LTB_4. Esse reconhecimento ativa respostas biológicas nas células, tais como degranulação de neutrófilos, fagocitose, produção de Espécies Reativas de Oxigênio (ROS), Oxido Nítrico (NO), citocinas como GM-CSF, TNF- α, IL-6 e IL-1β, além de quimiocinas como Proteína quimioatrativa de monócitos (MCP-1), quimiocina CXC Ligante 1 (CXCL1) e CXCL2 (BRANDT; SEREZANI, 2017; TODA; YOKOMIZO; SHIMIZU, 2002).

No entanto, o impacto dessa inflamação estéril no desfecho da COVID-19 ainda é pouco conhecido, tornando necessários estudos com objetivo de compreender a imunopatogênse da doença para minimizar o dano e otimizar o cuidado clínico desses indivíduos.

1.2.6 **Suscetibilidade a infecções**

Apesar do estado inflamatório crônico, sistêmico e exacerbado, indivíduos com diabetes são mais suscetíveis a diferentes doenças infecciosas (ALVES; CASQUEIRO; CASQUEIRO, 2012a; BENFIELD; JENSEN; NORDESTGAARD, 2007; BONYEK-SILVA et al., 2020,

2021; BRANDT et al., 2018; KRAKAUER, 2015). Os estudos indicam que a hiperglicemia influencia o desfecho de doenças infecciosas independentemente do tipo de microrganismo. Já foi amplamente descrito que o diabetes piora quadros infecciosos causados por bactérias, fungos, vírus e até mesmo protozoários (ALVES; CASQUEIRO; CASQUEIRO, 2012b; BONYEK-SILVA et al., 2020, 2021; CHÁVEZ-REYES et al., 2021). Juntamente com um comprometimento na indução de EROs, a produção de mediadores inflamatórios de forma aberrante ou desbalanceada vem sendo associada com o aumento da suscetibilidade em diferentes doenças infecciosas no contexto do diabetes (BONYEK-SILVA et al., 2020; BRANDT et al., 2018).

Estima-se que cada 1 mmol/L de glicose acima dos níveis normais aumenta cerca de 6-10% o risco para infecções de pele, do trato urinário e no tecido pulmonar (BENFIELD; JENSEN; NORDESTGAARD, 2007). Assim, a COVID-19, uma doença de caráter infeccioso, agravada por uma inflamação exacerbada e em um tecido sensível ao dano, acende um alerta em indivíduos mais suscetíveis como os portadores do diabetes.

1.2.7 **Diabetes e a COVID-19**

A inflamação crônica de baixo grau, o aumento da suscetibilidade a infecções e o agravamento de doenças pulmonares estão entre as complicações mais negligenciadas dentre aquelas que acometem indivíduos diabéticos. Considerando a importância da elucidação dos mecanismos envolvidos nestas complicações, a pandemia da COVID-19 tornou urgente essa questão para o diabetes, um importante fator de risco para a forma grave da doença.

Desde o início da pandemia da COVID-19 a comunidade médica já relatava a grande quantidade de pacientes com diabetes admitidos nos hospitais devido as complicações da infecção pelo SARS-CoV-2 (BONYEK-SILVA et al., 2021; RICHARDSON et al., 2020; WANG et al., 2020b). No entanto, ainda eram desconhecidos os fatores que predispunham alguns indivíduos a desenvolver a forma grave da doença. Atualmente, alguns mecanismos já são compreendidos, tais como o aumento na expressão da ECA2, o principal receptor para infecção do SARS-CoV-2, em células mononucleares do sangue periférico (PBMCs) cultivadas em condições de hiperglicemia. Além disso, o aumento na produção de IL-1b, IL-6 e TNF-a observado após a infecção de maneira dependente da concentração de glicose (CODO et al., 2020). Este aumento na expressão de ECA2 juntamente com as citocinas, em PBMCs sob condições hiperglicêmicas, foi acompanhado de menor eliminação do SARS-CoV-2 (CODO et

al., 2020). Porém, atualmente é consenso na literatura que o diabetes, apesar de ter a hiperglicemia como característica base, é uma doença complexa, envolvendo diferentes fatores biológicos, desde alteração hormonal até danos vasculares. Essa alta complexidade dificilmente é levada em consideração em modelos de cultura celular de doadores saudáveis. Assim, Bonyek-Silva e colaboradores demonstraram que PBMCs de pacientes com diabetes acometidos pela COVID-19, de fato, possuem um aumento na expressão dos genes que codificam ECA2, mas também de TMPRSS2 e LTB_4. Estas alterações estavam envolvidas na gravidade da doença nesses pacientes (BONYEK-SILVA et al., 2021).

Nosso estudo demonstrou que pacientes diabéticos com COVID-19 requerem mais tempo de internação em leitos clínicos e de UTI, além da duração da doença ser maior nesses indivíduos. Ademais, a capacidade de troca gasosa nesses pacientes foi mais comprometida em comparação aos indivíduos sem diabetes (BONYEK-SILVA et al., 2021). Achados similares já foram descritos ao analisar os pulmões, principal órgão afetado pela COVID-19, de pacientes com diabetes sem infecção pelo SARS-CoV-2, onde apresentam aumento da proteína ECA2, podendo também influenciar o desfecho da doença (WIJNANT et al., 2020). A gravidade da doença também é influenciada pela hiperglicemia, podendo ser um marcador desde a admissão nos hospitais dos pacientes por COVID-19. No entanto, o manejo e controle glicêmico desses pacientes durante a fase aguda da COVID-19 foi marcada por incertezas. A utilização de normoglicemiantes, como a Metformina, foi associada à piora do quadro clínico desses pacientes, porém sem aumentar a taxa de mortalidade (CHENG et al., 2020). Por ser um doença recente, esses achados ainda não são consenso entre a comunidade científica (BRAMANTE et al., 2021), uma vez que outras evidências sugerem que a insulina e inibidores de DPP4 são estratégias mais eficazes para serem utilizadas em diferentes formas clínicas da doença (LIM et al., 2021; SARDU et al., 2020; WANG et al., 2020b).

Assim como o diabetes, outros fatores de risco vêm sendo estudados por diferentes grupos de pesquisas, tais como o pré-diabetes. Alguns poucos relatos na literatura e perspectivas individuais indicam que esses pacientes com COVID-19 parecem evoluir de forma diferente, uma vez que não apresentam prognóstico como indivíduos sem diabetes, mas também não agravam como os portadores do diabetes (HEIDARPOUR et al., 2020; KOH et al., 2021; SATHISH; CHANDRASEKARAN, 2021; SMITH et al., 2021; SOURIJ et al., 2021; WANG et al., 2020b). Nesse contexto, Wang e colaboradores mostrou o impacto do nível glicêmico em condições normais, de pré-diabetes e diabetes na mortalidade e agravamento de pacientes com

COVID-19, onde foi observado que existia uma relação de aumento do nível glicêmico e o pior prognóstico para a doença (WANG et al., 2020b).

O conhecimento sobre a associação entre o diabetes e a COVID-19 durante a fase aguda da infecção pelo SARS-CoV-2 segue avançando, apesar dos estudos sobre esse tema ainda serem escassos. Ademais, muitos relatos na literatura tem abordado o surgimento de sequelas após a fase aguda da doença, como fadiga, cefaleia, déficit de atenção, queda de cabelo e dispneia, as quis podem persistir por meses (LOPEZ-LEON et al., 2021). Apesar do diabetes ser relatado em pacientes que apresentaram sintomas residuais da COVID-19 por longo período, o diabetes não parece sem um grande preditor para o aparecimento dessas sequelas, uma vez que a o perfil de sintomas relatados não difere daqueles de pacientes que não possuem diabetes (FERNÁNDEZ-DE-LAS-PEÑAS et al., 2021; SUDRE et al., 2021).

2 **JUSTIFICATIVA**

A pandemia da COVID-19 tem provocado internações e óbitos por todo o mundo. Com uma capacidade de transmissão superior à dos demais coronavírus, o SARS-CoV-2 tem infectado milhões de pessoas, que frequentemente já possuem outras comorbidades, dentre elas, as doenças metabólicas, como obesidade, hipertensão e diabetes.

O diabetes, causado por elevados níveis de glicose na circulação sanguínea, é uma das doenças metabólicas com grande incidência em todo o mundo. Dentre outras complicações, os indivíduos portadores do diabetes naturalmente possuem uma inflamação crônica de baixo grau persistente e um aumento na suscetibilidade a infecções. Estas condições podem aumentar o risco de evolução para a forma grave da COVID-19, uma doença infecciosa e de caráter inflamatório. Assim, desde os primeiros relatos sobre a COVID-19, o diabetes foi incluído como um dos principais fatores de risco para o agravamento da doença. No entanto, os possíveis mecanismos envolvidos nesse aumento de risco para a forma grave da COVID-19 nessa população ainda são pouco conhecidos. Portanto, estudos que busquem identificar, descrever e explorar esses mecanismos com a finalidade de minimizar os impactos socioeconômicos da doença são de grande importância para a atual e possíveis futuras pandemias decorrentes de infecções virais.

3 **MANUSCRITO I 3**

3.1 HIPÓTESE

A inflamação exacerbada em indivíduos com diabetes piora o desfecho da COVID-19.

3.2 OBJETIVOS

3.2.1 **Objetivo geral**

Identificar mecanismos imunológicos associados ao agravamento da COVID-19 em indivíduos com diabetes.

3.2.2 **Objetivos específicos**

- Analisar o perfil de expressão gênica em PBMCs induzido pelo diabetes utilizando dados globais públicos;
- Identificar vias de sinalização induzida pelo diabetes associadas ao agravamento da COVID-19;
- Validar os achados de expressão gênica em PBMCs de pacientes diagnosticados com COVID-19 e diabetes;
- Mensurar os níveis de mediadores inflamatórios no plasma de pacientes com diabetes ou não, ambos diagnosticados com COVID-19;
- Correlacionar os achados de expressão e mediadores inflamatórios com a gravidade da COVID-19 em pacientes com diabetes ou não;
- Caracterizar a população e o estado clínico de pacientes com diabetes ou não, ambos admitidos em hospital de referência ao tratamento para COVID-19 na cidade de Salvador – BA.

3.3 LTB4-DRIVEN INFLAMMATION AND INCREASED EXPRESSION OF ALOX5/ACE2 DURING SEVERE COVID-19 IN INDIVIDUALS WITH DIABETES

Inflamação guiada por LTB_4 e aumento da expressão de ALOX5/ACE2 durante a COVID-19 em indivíduos com diabetes

O objetivo desse estudo foi avaliar se a inflamação crônica de baixo grau do diabetes poderia desempenhar um papel no desenvolvimento da COVID-19 grave. Para isso, coletamos dados clínicos e amostras de sangue de pacientes com e sem diabetes internados por COVID-19. As amostras de plasma foram usadas para dosar mediadores inflamatórios e as células mononucleares do sangue periférico foram destinadas para análise de expressão gênica do receptor principal para o SARS-CoV-2 (ACE2/TMPRSS2) e da molécula principal da síntese do leucotrieno B_4 (LTB_4), codificada pelo gene ALOX5. Os achados mostram que o diabetes induz a ativação da via do LTB_4 e que durante a COVID-19 aumenta a expressão de ACE2/TMPRSS2 e ALOX5. O diabetes também foi associado a distúrbios relacionados a COVID-19, como redução da saturação de oxigênio e aumento na duração da doença. Além disso, a expressão aumentada de ACE2 e ALOX5 foi relacionada com a gravidade da doença nesses pacientes. Este estudo confirmou que um dos produtos da via dos leucotrienos, o LTB_4, estava significativamente aumentado em nível plasmático nos indivíduos com diabetes. Além disso, podemos verificar que os níveis séricos de IL-6 estavam aumentados apenas em indivíduos com diabetes que necessitaram de cuidados intensivos. Em conjunto, esses resultados indicam que os níveis sistêmicos de LTB_4 e IL-6, bem como a expressão de ACE2/ALOX5 nas células do sangue, podem estar associados com a gravidade da COVID-19 em indivíduos com diabetes.

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LTB_4-Driven Inflammation and Increased Expression of *ALOX5/ACE2* During Severe COVID-19 in Individuals With Diabetes

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COMPLICATIONS

Diabetes is a known risk factor for severe coronavirus disease 2019 (COVID-19), the disease caused by the new coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). However, there is a lack of knowledge about the mechanisms involved in the evolution of COVID-19 in individuals with diabetes. We aimed to evaluate whether the chronic low-grade inflammation of diabetes could play a role in the development of severe COVID-19. We collected clinical data and blood samples of patients with and without diabetes hospitalized for COVID-19. Plasma samples were used to measure inflammatory mediators and peripheral blood mononuclear cells, for gene expression analysis of the SARS-CoV-2 main receptor system (*ACE2/TMPRSS2*), and for the main molecule of the leukotriene B_4 (LTB_4) pathway (*ALOX5*). We found that diabetes activates the LTB_4 pathway and that during COVID-19 it increases *ACE2/TMPRSS2* as well as *ALOX5* expression. Diabetes was also associated with COVID-19–related disorders, such as reduced oxygen saturation as measured by pulse oximetry/fraction of inspired oxygen (FiO_2) and arterial partial pressure of oxygen/FiO_2 levels, and increased disease duration. In addition, the expressions of *ACE2* and *ALOX5* are positively correlated, with increased expression in patients with diabetes and COVID-19 requiring intensive care assistance. We confirmed these molecular results at the protein level, where plasma LTB_4 is significantly increased in individuals with diabetes. In addition, IL-6 serum levels are increased only in individuals with diabetes requiring intensive care assistance. Together, these results indicate that LTB_4 and IL-6 systemic levels, as well as *ACE2/ALOX5* blood expression, could be early markers of severe COVID-19 in individuals with diabetes.

As of 17 May 2021, >162 million confirmed cases of coronavirus disease 19 (COVID-19) and >3.3 million deaths worldwide from the pandemic had been recorded (1). The disease is caused by the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that emerged in China and rapidly spread around the world (2). Estimates indicate that ~80% of infected individuals are asymptomatic or develop mild symptoms. The other 20% can develop moderate to severe disease, occasionally

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requiring medical assistance due to acute respiratory disease and pneumonia, burdening health care systems (3,4). Risk factors in developing severe COVID-19 include, among others, hypertension, age, obesity, and diabetes (5–9). Individuals with diabetes are at high risk of developing severe COVID-19 as accounted for by their high rates of intensive care unit (ICU) admission and death (7).

Considering that 463 million people live with diabetes worldwide (10) and that COVID-19 is a highly transmissible disease, the need for identification of mechanisms that prevent infection in this population is urgent (6,7). As seen in multiple infectious diseases, including COVID-19, infection-induced inflammatory response can result in a cytokine storm, recruiting cells to infected tissues and establishing a proinflammatory feedback loop. This uncontrolled inflammation causes multiorgan damage, especially of the heart, liver, and kidneys, with high risk of death (11). Although several reports have described cytokines and chemokines involved in the inflammatory storm during COVID-19 (11,12), studies on lipid mediators of inflammation and their roles in this new disease are scarce.

Eicosanoids are potent lipid mediators produced by arachidonic acid metabolism, found in cell surface, that signals many biological processes, including inflammation and immune responses (13). Some classes of eicosanoids, especially leukotrienes, have been associated with the pathogenesis of respiratory disease (14,15). We and others have already shown increased levels of leukotriene B_4 (LTB_4) in diabetes, which is associated with inflammation, compromised wound healing, insulin resistance, and susceptibility to infections (16–20). LTB_4 is a product of the action of 5-lipoxygenase (5-LO) (encoded by the arachidonate 5-lipoxygenase [*ALOX5*] gene) and its activating protein FLAP (encoded by *ALOX5AP* gene) that are rapidly produced after several stimuli, mainly by neutrophils and monocytes/macrophages. After its release, LTB_4 can be signaled in an autocrine or paracrine manner by different cell types through the leukotriene receptor (encoded by the *LTB4R* gene), triggering an increase in chemotaxis and inflammatory exacerbation (18,21–23). In the current study, we sought to evaluate whether LTB_4 plays a role in the severity of COVID-19 in individuals with diabetes.

RESEARCH DESIGN AND METHODS

Ethics Statement

This study followed the principles specified in the Declaration of Helsinki. The Institutional Board for Ethics in Human Research at the Gonçalo Moniz Institute (Oswaldo Cruz Foundation) and Irmã Dulce Social Works approved this study (protocol numbers CAAE 36199820.6.0000.0040 and 33366020.5.0000.0047, respectively). Participants gave informed consent previous to any data and sample collection.

Acquisition of Microarray Data Set

Diabetes is considered a risk factor for complicated acute respiratory syndrome caused by SARS-CoV-2 infection (5,7). Given the lack of data on the mechanisms that drive these complications, we sought to analyze public transcriptome data of peripheral blood mononuclear cells (PBMCs) from individuals with diabetes. Microarray analysis was performed from a search of the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database using the terms "diabetes" and "human." Among the data sets found, we selected the data set with GEO accession number GSE95849 that was done on Phalanx Human lncRNA OneArray v1_mRNA (GPL22448) platform (24). This data set compared six samples of PBMCs from healthy control subjects (individuals with normal glucose tolerance and without a family history of diabetes or chronic diseases) and six samples from individuals with diabetes. The criteria for including individuals in the diabetes group were fasting plasma glucose $\geq$7 mmol/L, 2-h plasma glucose after oral glucose tolerance test $\geq$11.1 mmol/L, or use of glucose-lowering drugs or physician-diagnosed diabetes. Differentially expressed genes (DEGs) were considered when the fold change ranged from -2.0 to 2.0 with a false discovery rate–adjusted $P < 0.05$.

Detection of Metabolic Network in Diseases and Pathway Enrichment Analysis

Metabolic networks (compound-reaction-enzyme-gene) were found based on the expression of significantly modulated genes in comparisons of healthy control subjects with individuals with diabetes. We used MetDisease version 1.1.0 in Cytoscape 3.7.2 software (Cytoscape Consortium, San Diego, CA) to build disease-based metabolite networks according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). Next, data were further filtered to retain disease Medical Subject Headings terms relevant to reported clinical COVID-19 manifestations, such as pneumonia, respiratory distress syndrome (adult), acute lung injury, and inflammation. Matched metabolites found in these conditions were clustered using a Venn diagram to find common molecules.

The identification of enriched pathways was based on genes and compounds using the integrated KEGG and Edinburgh Human Metabolic Network (EHMN) databases stored at the National Center for Biotechnology Information. Canonical pathways were detected by MetScape version 3.1.3 in the Cytoscape 3.7.2 software using significantly modulated genes between healthy control subjects and individuals with diabetes.

Study Design, Cohort Definition, and Clinical Data

Patients were admitted with confirmed diagnosis of COVID-19 at Ernesto Simões Filho General Hospital, Salvador, Bahia, Brazil. A convenience sample of 53 patients were enrolled in this study (24 without diabetes [the non-DM group: NDM] and 29 with diabetes [the diabetes

mellitus group: DM]). This sample size considered a 95% CI (two-sided), and the power estimated for each parameter measured, using Epi Info software, was >80%. All groups were matched for sex, age, and hospitalization type (i.e., clinical beds [CBs], ICU). According to the Brazilian Diabetes Society guidelines, 2019–2020 (25), the diagnosis of diabetes was confirmed by HbA_{1c} levels measured during hospitalization. Patients with HbA_{1c} ≥6.5% (48 mmol/mol) and a medical history of insulin use were considered to have diabetes. The NDM group included individuals with HbA_{1c} ≤6.4% (46 mmol/mol) who were not considered to have diabetes or prediabetes (without the need for insulin during hospitalization). Comorbidity data were collected according to medical records. The study included patients with a positive diagnosis of COVID-19 based on positive molecular test (quantitative real-time PCR), serology or tomography results for or clinical history of COVID-19. Patients who did not agree to sign the free and informed consent, were pregnant, had symptoms for ≥14 days, and had been in the hospital for >48 h were excluded. Clinical data from all patients, obtained from medical records, are shown in Table 1.

Sample Collection

Blood samples from all patients were collected at admission by venipuncture using tubes with heparin. Plasma was separated (to quantify inflammatory mediators), and PBMCs (to analyze gene expression) were purified using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO).

Analysis of Gene Expression in PBMCs

Total RNA was extracted from PBMCs using miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's guidelines. Relative expression of *ALOX5* (assay identifier [ID] Hs.PT.56a.28007202.g); *ACE2* (assay ID Hs.PT.58.27645939); transmembrane serine protease 2 (*TMPRSS2*) (assay ID Hs.PT.58.4661363); furin, paired basic amino acid cleaving enzyme *(FURIN)* (assay ID Hs.PT.58.1294962), and basigin (*CD147*) (assay ID Hs.PT.56a.39293590.g) were analyzed. After RNA quantification and quality analysis by spectrophotometry, cDNA synthesis was performed using the SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). Then, cDNA was amplified by quantitative real-time PCR using the SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). Relative gene expression is shown as the fold change between the NDM and DM groups using the $2^{-\Delta\Delta CT}$ method [ΔΔCt = ΔCt (target DM) − mean ΔCt (target NDM), where ΔCt = Ct (gene of interest) − Ct (housekeeping gene)]. To identify the distribution within the control group (NDM), we applied ΔΔCt = ΔCt (target NDM) − mean ΔCt (target NDM), with ΔCt = Ct (gene of interest) − Ct (housekeeping gene). β-Actin was the housekeeping gene (*ACTB*) (assay ID Hs.PT.39a.22214847). All primers were purchased from Integrated DNA Technologies (Coralville, IA).

Quantification of Inflammatory Mediators

Based on the inflammatory profile already described in the literature for diabetes and COVID-19 (6,8,26), serum levels of TNF-α, IL-6, and IL-1β cytokines

Table 1—Characteristics of individuals hospitalized because of complications of COVID-19, Salvador, Bahia, Brazil, 2020 (*N* = 53)

	NDM	DM	*P*
Patients, *n*	24	29	
Male sex, *n* (%)	15 (62.5)	16 (55)	0.59
Age (years), median (min–max)	59 (27–88)	59 (43–93)	0.12
HbA_{1c}, median (min–max)			<0.0001
%	5.6 (4.5–6.3)	7.9 (6.5–12.9)	
mmol/mol	38 (26–45)	63 (48–117)	
Comorbidities, *n/N* (%)			
Obesity	3/18 (16.6)	7/21 (33.3)	0.23
Dyslipidemia	3/13 (23.0)	3/11 (27.2)	0.99
Liver disease	1/22 (4.5)	0/24 (0.0)	0.47
Kidney disease	8/24 (33.3)	5/27 (18.5)	0.22
COPD	3/16 (18.7)	3/14 (21.4)	0.99
HAS	9/24 (37.5)	22/26 (84.6)	0.001
Symptoms, *n/N* (%)			
Fever	12/19 (63.1.0)	14/21 (66.6)	0.99
Cough	16/23 (69.5.5)	16/22 (72.7)	0.81
Dyspnea	13/22 (59.0)	22/24 (91.6)	0.01
Expectoration	1/17 (5.8)	3/16 (18.7)	0.33
COVID-19 confirmed, *n/N* (%)	18/21 (85.7)	26/27 (96.3)	0.30

HAS, systemic arterial hypertension; min–max, minimum to maximum; *n/N* positive number/valid number.

(Invitrogen) were evaluated using sandwich ELISAs. LTB_4 levels were determined by Competition ELISA Kit (Cayman Chemical, Ann Arbor, MI), considering the manufacturer's instructions.

Statistical Analysis

The Benjamini-Hochberg method was used to control false discovery rate in evaluation of DEGs from the GEO transcriptome data set. For variables with normal distribution, we used Student *t* test (two groups) and one-way ANOVA test followed by Tukey post hoc test (three or more groups). For nonnormal distribution, we used Mann-Whitney test (two groups), Kruskal-Wallis with Dunn posttest (three or more groups), and Spearman test for correlation analysis. Symptom and comorbidity analyses were performed using χ^2 or Fisher exact test. All tests were conducted using GraphPad Prism 7 software (GraphPad Software, San Diego, CA). Differences were considered statistically significant when $P < 0.05$ or adjusted $P < 0.05$ for DEGs and multiple comparisons.

Data and Resource Availability

The public data set analyzed during the current study is available in GEO under accession number GSE95849 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE95849). The data sets generated during the current study are not publicly available but can be made available by the corresponding author upon request.

RESULTS

LTB_4 Signaling Activated in Individuals With Diabetes Is Similar to That Found in Respiratory Disorders

Initially, we found that 3,585 genes were significantly modulated when comparing cells from individuals with or without diabetes. Of these, 3,405 were upregulated, and 180 were downregulated in individuals with diabetes (Fig. 1*A*).

Next, we searched for disorders associated with these DEGs by detecting molecule networks. We focused on conditions related to severe COVID-19, such as pneumonia, severe acute respiratory syndrome, and acute lung injury; we also focused on inflammation. Interestingly, we found only two molecules in common among these conditions: carbon dioxide and the lipid mediator LTB_4 (Fig. 1*B*).

We further searched for signaling pathways associated with these DEGs, and among 61 routes found, the LTB_4 pathway was at a central position within the network (Fig. 1*C*). Next, we assessed the expression of molecules crucial for LTB_4 production, such as the *ALOX5* gene (which encodes the 5-LO enzyme that converts arachidonic acid into leukotrienes), *ALOX5AP* (the 5-LO–activating protein), and *LTB4R* (the LTB_4 receptor) in this data set. We found increased expression of all evaluated genes in the PBMCs from DM compared with NDM (Fig. 1*D*). Together, these findings indicate that LTB_4 is a potential target to study mechanisms under complicated COVID-19 in individuals with diabetes.

Increased Expression of *ALOX5* and *ACE2/TMPRSS2* in PBMCs From DM and NDM Patients With COVID-19

The expression of SARS-CoV-2 receptors (27) and the inflammatory response (11) are related to the complications found in COVID-19. We then assessed the expression of *ALOX5* (which encodes for the 5-LO enzyme) and *ACE2/TMPRSS2*, *FURIN*, and *CD147* (surface molecules used by SARS-CoV-2 to invade human cells). The results showed a significant increase in the expression of *ALOX5* (Fig. 2*A*) and *ACE2/TMPRSS2* (Fig. 2*B* and *C*) in PBMCs from COVID-19 in DM compared with NDM. The increase in *ALOX5*, *ACE2*, and *TMPRSS2* was also preliminarily assessed in the tracheal secretion of the NDM and DM groups with COVID-19 under mechanical ventilation. Despite the small sample size, we observed a trend toward increased expression, indicating that blood cells mirror the immune response in the lungs ($P = 0.055$) (Supplementary Fig. 1). These findings confirm our previous result (from public transcriptome data), showing that *ALOX5* expression is increased in diabetes (Fig. 1*D*). Such findings support the possible role of 5-LO in the chronic low-grade inflammation observed in LTB_4 pathway–induced diabetes, rendering individuals with diabetes more prone to infections (19,21). We also found increased expressions of the SARS-CoV-2 main receptor system *ACE2* and *TMPRSS2* in the PBMCs from individuals in the DM group, suggesting that immune cells that will fight the infection are more prone to viral invasion.

Expression of *ALOX5* Correlates With That of *ACE2* in PBMCs From DM Patients With COVID-19

ACE2 expression is crucial for cell invasion and progression in COVID-19 (11,27). Therefore, we investigated whether the expression of *ALOX* could be correlated with *ACE2* expression. First, we correlated *ALOX5* with *ACE2/TMPRSS2* (summarized in the correlation matrix [Fig. 3*A*]) separately between the DM and NDM groups. We found a positive correlation between *ACE2* and *TMPRSS2* in both groups since these molecules act together during viral invasion (11) (Fig. 3*B* and *C*). However, the correlation between *ALOX5* and *ACE2* was only present in the DM group (Fig. 3*D* and Supplementary Fig. 2), suggesting that cells that have high levels of *ALOX5* also have increased *ACE2* expression in the DM group.

Next, we evaluated whether *ALOX5* and *ACE2* expressions are correlated with the clinical evolution of COVID-19. First, we compared the need for ICU admission between the DM and NDM groups stratified by the expression levels of *ALOX5* and *ACE2*. The results showed that individuals in the DM group with higher levels of *ACE2* (Fig. 3*E*) and *ALOX5* (Fig. 3*F*) required ICU care more frequently than those with low expression of these genes, but no difference was found with the gene expression of *TMPRSS2* (Fig. 3*G*). Together, these findings

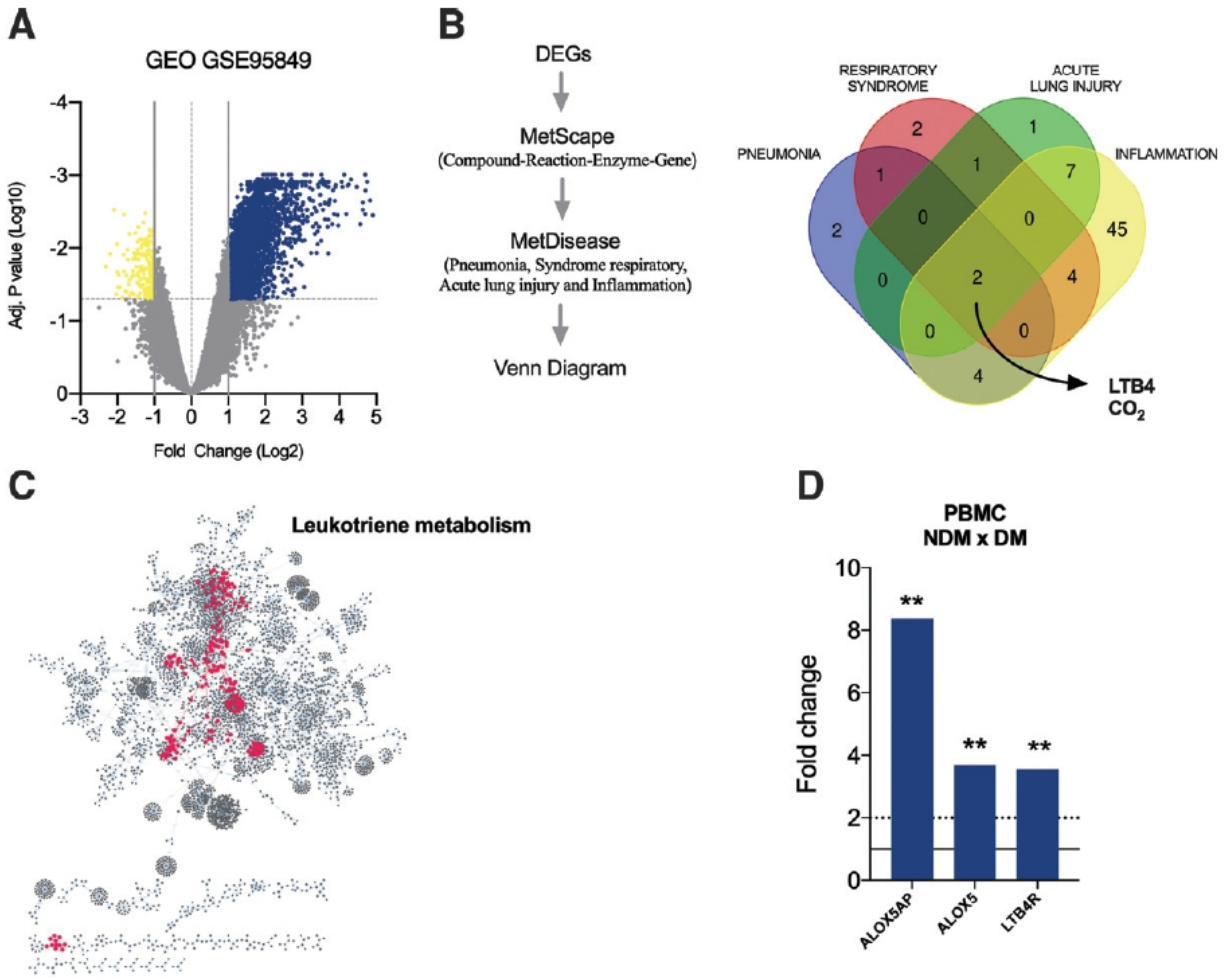

Figure 1—Upregulation of LTB_4 signaling in individuals in the DM group. *A*: Volcano plot with DEGs (blue, upregulated genes; yellow, downregulated genes) in PBMCs from DM patients vs. NDM patients. *B*: Workflow to identify molecules associated with inflammation and respiratory disorders based on gene expression shown in A and the resulting Venn diagram showing molecules in common among pneumonia, respiratory syndrome, acute lung injury, and inflammation. *C*: Enriched pathways raised from DEG analyses of PBMCs from DM patients vs. NDM patients, highlighting in red the central position of leukotriene metabolism among pathways. *D*: Fold change of genes involved with LTB_4 production (*ALOX5AP* and *ALOX5*) and signaling (*LTB4R*) in PBMCs of DM vs. NDM patients. Dotted line = cutoff point for a DEG; solid line = average of the control group. Data are medians. $^{**}P < 0.01$. Adj., adjusted.

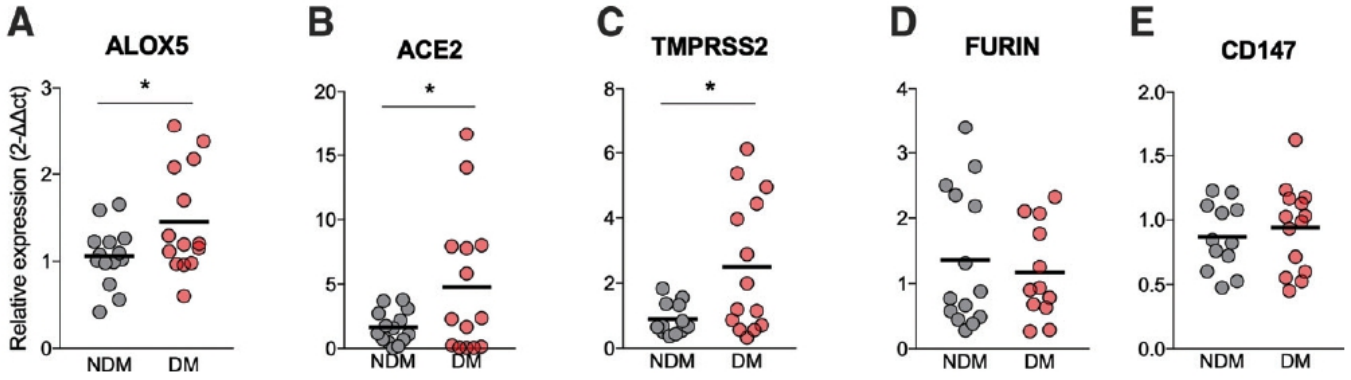

Figure 2—Increased expression of ALOX5 and ACE2/TMPRSS2 receptor system for SARS-CoV-2 infection in individuals with COVID-19 in the DM group. Expressions of *ALOX5* (*A*), *ACE2* (*B*), *TMPRSS2* (*C*), *FURIN* (*D*), and *CD147* (*E*) in PBMCs from DM and NDM patients. Data are means. $^{*}P < 0.05$, $^{**}P < 0.01$.

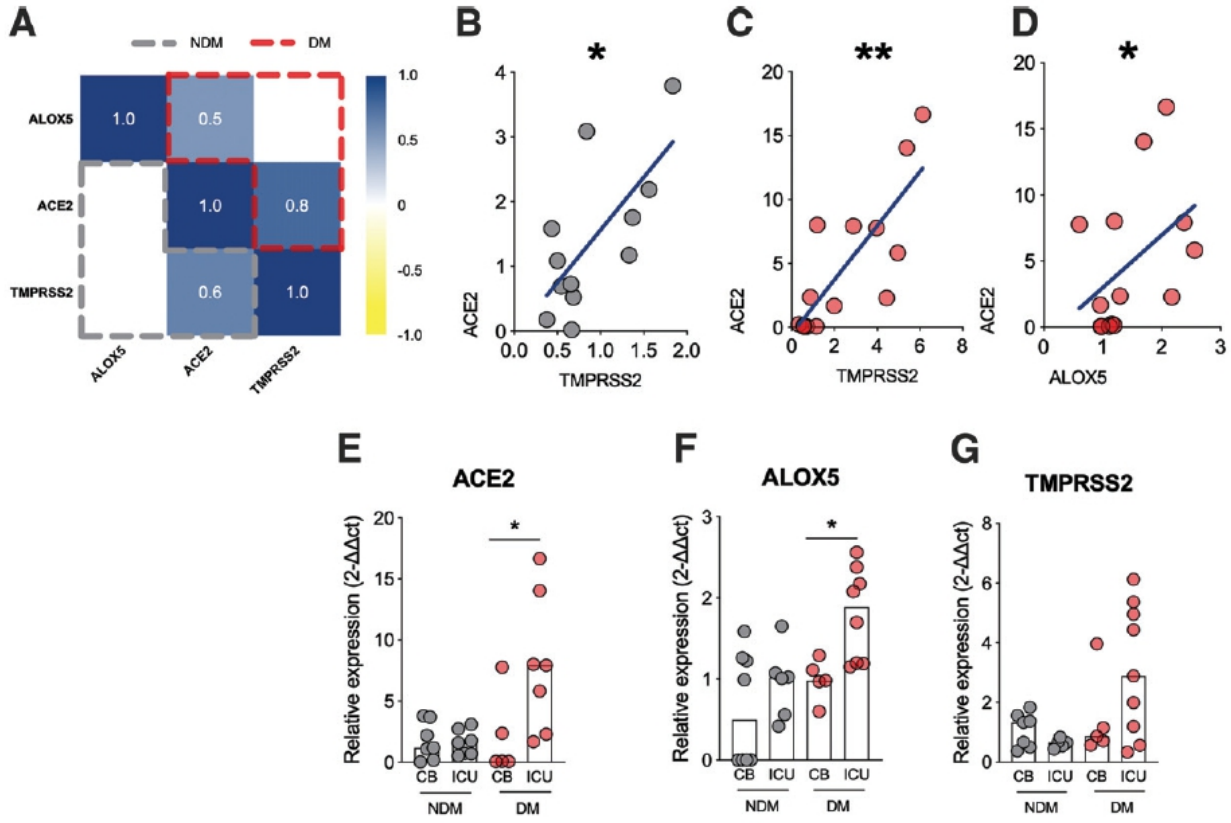

Figure 3—ALOX5 expression positively correlates with ACE2 expression in individuals with diabetes and COVID-19, and this is associated with an increased rate in ICU admissions. *A*: Correlation matrix between *ALOX5* and *ACE2*/*TMPRSS2* expression in PBMCs from DM (red) and NDM (gray) patients. *B*: Correlation analysis between ACE2 and TMPRSS2 expressions in PBMCs from of NDM (*B*) and DM (*C*) individuals with COVID-19. *D*: Correlation analysis between *ALOX5* and *ACE2* expression in PBMCs from DM patients. *E* to *G*: Hospitalization type among DM or NDM individuals with COVID-19 based on the expression of *ACE2*, *ALOX5* and *TMPRSS2*. Data are medians. Spearman *r* correlation. $*P < 0.05$, $**P < 0.01$.

indicate that the increased expressions of *ALOX5* and *ACE2* in blood cells from individuals with diabetes are associated with more severe conditions of COVID-19, requiring ICU care.

Increased Systemic Levels of LTB_4 in DM Patients With COVID-19

The cytokine storm described in COVID-19 is characterized by several inflammatory mediators. However, the role of lipid mediators in this context is still unknown (11). We measured the levels of inflammatory cytokines (IL-6, TNF-α, and IL-1β) and a lipid mediator of inflammation (LTB_4) in the plasma of individuals with and without diabetes and COVID-19. The results showed a significant increase of LTB_4 levels in the sera of individuals in the DM group (Fig. 4*A*). No statistical differences in the levels of IL-6 (Fig. 4*B*), TNF-α (Fig. 4*C*), or IL-1β (Supplementary Fig. 3) were found in comparisons of the DM and NDM groups. Supplementary Fig. 4 shows the production of these inflammatory mediators individually for each patient in the NDM and DM groups.

We further detailed the production of LTB_4, IL-6, and TNF-α between the NDM and DM groups based on the hospitalization type. No differences were found for LTB_4 and TNF-α production (Fig. 4*D* and *F*). With regard to IL-6 production, in the DM group, there was a significant increase in ICU admissions compared with CB admissions (Fig. 4*E*). Together, these findings indicate the predominance of LTB_4 production in the DM group compared with the NDM group. Moreover, IL-6 production seems to be an indicator for COVID-19 severity (hospitalization type) in the DM group.

ALOX5 Expression, Involved in LTB_4 Synthesis, Was Correlated With Clinical Outcomes of COVID-19 in the DM Group

Despite studies reporting diabetes as a risk factor for COVID-19, few explored the mechanisms related to these patients' worse prognosis (7,28,29). We compared LTB_4 signaling in patients with different clinical outcomes associated with COVID-19. In an analysis of days spent in the hospital (Fig. 5*A*) and death rate (Fig. 5*B*), we found no difference between the NDM and DM groups. However, there was a significantly longer disease duration (the period between symptom onset and disease outcome [death or hospital discharge]) in the DM group (Fig. 5*C*). These data suggest that individuals with diabetes develop COVID-19 symptoms for prolonged periods, possibly due to the low-

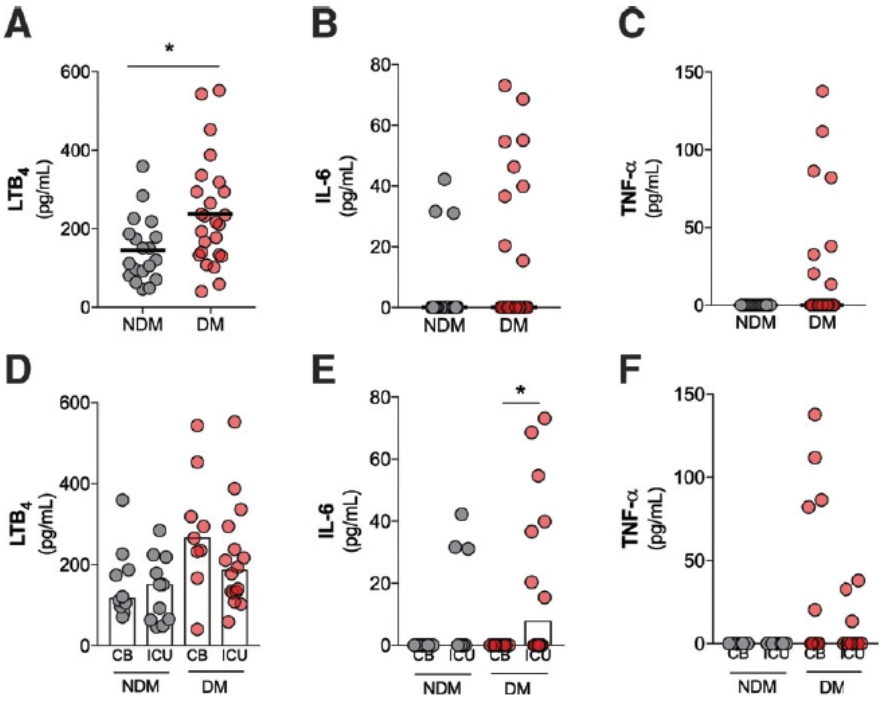

Figure 4—Increased systemic levels of LTB_4 in patients in the DM group, with COVID-19. Levels of LTB4 (*A*), IL-6 (*B*), and TNF-α (*C*) in plasma samples from DM and NDM patients affected by COVID-19. Plasma levels of LTB4 (D), IL-6 (E), and TNF-α (F) in NDM and DM patients with COVID-19 categorized by hospitalization type: clinical beds (CB) or intensive care units (ICU). Data are means. *$P < 0.05$.

grade inflammation already present in these individuals even in the absence of an infectious agent. Furthermore, the pulmonary condition in the DM group was more severe than in the NDM group, measured by oxygen saturation by pulse oximetry (SpO_2)–to–fraction of inspired oxygen (FiO_2) ratio (Fig. 5*D*), arterial partial pressure of oxygen (PaO_2)–to–FiO_2 ratio (Fig. 5*E*), and O_2 saturation (Supplementary Fig. 5) at the moment of admission to the hospital. For both parameters, individuals in the DM group arrived at the hospital in a more critical condition.

Finally, we correlated these clinical aspects with LTB_4 production and *ALOX5* and *ACE2* expression in all individuals (Fig. 6*A*). The results show a positive correlation between LTB_4 and *ALOX5*, as expected, since the 5-LO enzyme produces LTB_4 (r = 0.5) (Supplementary Fig. 6*A*). We found that *ALOX5* negatively correlates with the worse pulmonary condition, such as SpO_2-to-FiO_2 ratio (r = −0.6) and PaO_2-to-FiO_2 ratio (r = −0.9) (Fig. 6*B* and *C*). In addition, we found that patients with a low SpO_2-to-FiO_2 ratio and increased production of IL-6 had a longer hospital stay for COVID-19 (Fig. 6*D*).

Taken together, these results show that patients with COVID-19 and diabetes develop a more pronounced systemic inflammatory response with the predominance of

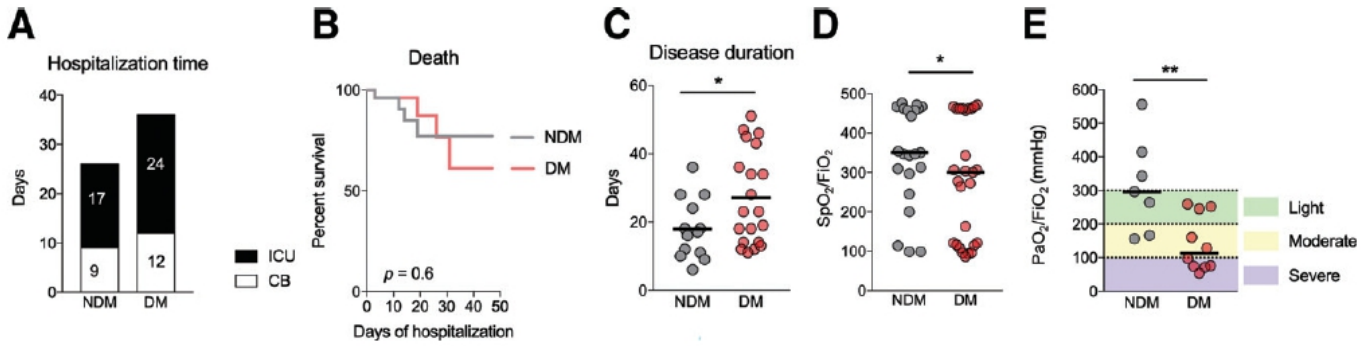

Figure 5—Diabetes induces greater severity of COVID-19. *A*: Number of days that NDM and DM patients remained hospitalized in CBs or the ICU because of COVID-19. *B*: Survival curves of NDM and DM patients hospitalized for COVID-19. *C*: Disease duration measured from the onset of symptoms to hospital discharge for NDM and DM patients with COVID-19. *D*: O_2 saturation of NDM and DM patients with COVID-19. *E*: Degree of lung injury in NDM and DM patients with COVID-19. Data are medians in *A*, *B*, and *D* and means in *C*. *$P <$ 0.05, **$P < 0.01$.

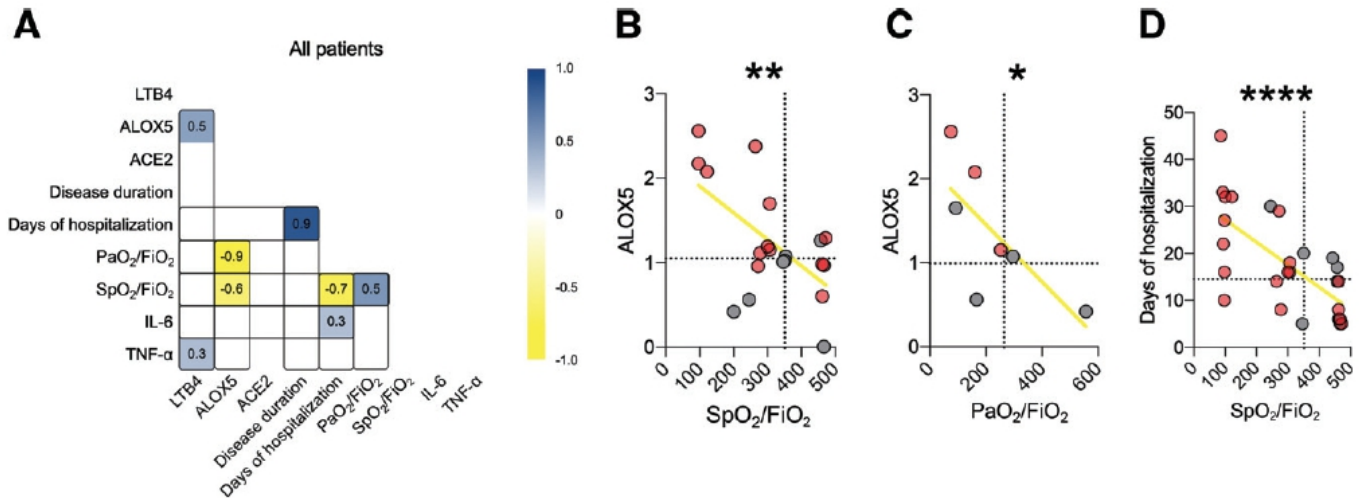

Figure 6—ALOX5 plays a role in the severity of COVID-19 in individuals with diabetes. *A*: Correlation matrix among genes, inflammatory parameters, and clinical outcome changes found in all patients with COVID-19. *B* and C: Dispersion of values with all patients between the correlation of ALOX5 with SpO_2-to-FiO_2 and PaO_2-to-FiO_2 ratios. *D*: Correlation between oxygen saturation and days of hospitalization. Dotted lines = median of the NDM group. Spearman *r* correlation. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$.

LTB_4 and increased expression of SARS-CoV-2 receptor system *ACE2/TMPRSS2*. These individuals more frequently require critical care due to lung injury, suggesting that LTB_4 signaling could be a mediator produced by individuals with diabetes that increases the risk for severe COVID-19.

DISCUSSION

As SARS-CoV-2 emerged and spread globally, identifying mechanisms involved in severe COVID-19 and its risk factors became crucial for improving disease management. Diabetes is considered a risk factor for severe COVID-19 (5,7,28), but the mechanisms under these complications remain unknown. Inflammation associates with severe COVID-19 (18,21,22), and LTB_4 drives the chronic low-grade inflammation observed in experimental models of diabetes, while its role is not fully elucidated in humans with diabetes (17–19,21,30–32). The current study shows that individuals with diabetes and COVID-19 have increased expression of genes from the LTB_4 pathway in blood cells. During COVID-19, the expression of *ACE2* and *TMPRSS2*, which encode the main receptor system for SARS-CoV-2 cell invasion, are also increased in PBMCs of individuals with diabetes. Moreover, the increased expression of *ALOX5* correlates with *ACE2*, which was present in patients with critical conditions requiring intensive care.

As revealed by pathway analysis, LTB_4 is critical in several physiological disorders (observed in severe COVID-19), including inflammation and respiratory complications such as pneumonia, respiratory distress syndrome, and acute lung injury (11,28). LTB_4 is also an essential molecule in diabetes pathogenesis. Several studies with experimental models have indicated that LTB_4 dictates the chronic low-grade inflammation in diabetes, rendering mice more prone to infections (17,19,33). Our group previously showed that increased production of LTB_4 induced by diabetes alters the outcome of cutaneous leishmaniasis (17). Another study showed that LTB_4 is associated with pulmonary complications, such as pneumonia, acute lung injury, acute respiratory distress syndrome (ARDS), and respiratory failure (15,34,35).

The interaction between SARS-CoV-2 and host cells involves several molecules, such as ACE2 and TMPRSS2 that interact with the viral spike protein (11,36,37). High glucose concentrations increase the expression of *ACE2* and SARS-CoV-2 viral load in human monocytes (27). A meta-analysis revealed an increase of *ACE2* expression in the lungs of patients with comorbidities, including diabetes (5), and another study showed an increase in the ACE2 protein in the lungs of individuals with diabetes (38). Besides the expression of ACE2 in the lungs, monocytes and lymphocytes are crucial for the COVID-19 immunopathogenesis (5,11,12,27,36). Our data show that *ACE2* and *TMPRSS2* expression are increased in PBMCs of individuals with diabetes and COVID-19, which can be related to a greater susceptibility to SARS-CoV-2 infection (27,38).

Additionally, *ALOX5* expression positively correlates with *ACE2*, and ICU admission is associated with increased *ALOX5/ACE2* expression in patients with diabetes and COVID-19. The interaction between the LTB_4 and ACE2 pathways is still unknown, but the positive independent regulation of these genes in monocytes can influence the process of inflammation and infection, respectively (21,27). During SARS-CoV-2 infection, mononuclear cells are recruited to the lung tissue, where they probably contribute to the control of infection and the healing process but also cause cause tissue damage (11).

In the current study, individuals in the DM group with COVID-19, age and sex matched to individuals in the NDM group with COVID-19, had a higher frequency of dyspnea, which is in agreement with data from Wuhan, China (7). Hypertension is more frequent in patients with diabetes and patients with COVID-19 and is a known risk factor for severe COVID-19 (7,38). According to previous studies, diabetes and hypertension are frequent in patients with COVID-19 and may play a role in increased death rates (6,7,39). In our study, mortality rates were similar between patients with COVID-19 with or without diabetes, but the disease severity is more pronounced in those with diabetes. Although our cohort shows no difference in obese individuals between the NDM and DM groups, the influence of weight differences between the groups should not be excluded, since obesity was determined only by medical observation.

The cytokine storm contributes to mortality in ~28% of fatal COVID-19 cases (11). This condition encompasses several cytokines and chemokines, such as IL-1β, IL-6, IFN-γ, MCP-1, CCL2, CXCL10, and TNF-α (11,28). The IL-6 cytokine is one of the most related to the severity of COVID-19, and as in previous studies, our findings demonstrate this association in the context of COVID-19 in individuals with diabetes (6,8,26). However, knowledge is lacking about the implications of lipid mediators in the inflammatory response during COVID-19. LTB_4 is a potent inducer of inflammatory cytokines, including those of the cytokine storm, which may drive COVID-19 severity (16,21). Bronchoalveolar lavage fluid exhibited high levels of LTB_4 in an experimental model of acute lung injury (34). LTB_4 plays a significant role in the chronic obstructive pulmonary disease (COPD), and individuals with severe COPD have high levels of LTB_4 in exhaled air; such levels correlate with disease severity (14). LTB_4 levels better correlate with lung injury severity and clinical outcomes in ARDS than several other eicosanoids (35).

The number of patients with severe COVID-19 who require ICU care is a challenge for health care systems worldwide. Individuals with ARDS exhibit three to five times more LTB_4 levels than control subjects (40). The role of LTB_4 in the outcome of lung diseases is associated with neutrophil tissue infiltration, a condition present in COVID-19 (12). Our group has recently shown that LTB_4 is involved in the activation of pathogen-induced inflammasomes (18). A recent preliminary study associated the activation of inflammasomes in the lungs of patients with COVID-19 with a worse disease prognosis (41).

The Randomized Evaluation of COVID-19 Therapy (RECOVERY) study showed that dexamethasone slightly reduced death rates among patients with COVID-19 requiring invasive mechanical ventilation or oxygen therapy (42). Additionally, montelukast, a leukotriene antagonist, is proposed for the prophylaxis of COVID-19 symptoms (43). Together, these studies suggested strategies to treat COVID-19 that, directly or indirectly, act through eicosanoids. Our results confirm that LTB_4 signaling is a crucial branch of the inflammatory response observed in COVID-19 and reinforces the possibility of its inhibition in clinical practice.

Several studies reported the association of diabetes and increased COVID-19 death rates (4,5,19,22), whereas others did not find such an association with disease severity (4,5,33). We have not found a direct association between diabetes and mortality rates in our cohort. The participants in the DM group in this study developed severe forms of COVID-19, requiring ICU hospitalization, but their disease evolution seemed similar to that of patients in the NDM group. On the other hand, we found a significantly longer disease duration in DM patients with COVID-19. The disease duration refers to the period between the onset of symptoms until the patient's discharge or death, indicating that patients with diabetes experience COVID-19 symptoms for prolonged periods.

Although we have not found a direct association between systemic levels of LTB_4 and a worse COVID-19 prognosis in individuals with diabetes, our findings show that patients with COVID-19 and diabetes more frequently present reduced SpO_2-to-FiO_2 and PaO_2-to-FiO_2 ratios that correlate with ALOX5 expression in the blood. The dissociation between the expression of the *ALOX5* gene and its metabolic product may be due to different sources of LTB_4 detected in the bloodstream. Different immune cell types are able to produce LTB_4, such as neutrophils (14), a cell type not represented in our sample of mononuclear cells. LTB_4 is also locally produced at the site of infection caused by different agents (17–19,44) and has been associated with increased lung injury in experimental models (34). Our results add a new player to the inflammation panorama of COVID-19, suggesting that circulating mononuclear cells already present a proinflammatory profile that, once recruited to the lung, may amplify local inflammation and tissue injury. Further studies are necessary to confirm pulmonary production of LTB_4 and its role in COVID-19 outcomes.

In summary, our findings show that diabetes induces a proinflammatory profile on circulating immune cells with increased expression of *ACE2* and *ALOX5* genes, rendering these cells more prone to SARS-CoV-2 invasion. Together, our data reveal a potential role of LTB_4 in COVID-19, which is poorly explored, and open new ways to study implications and applications of this mediator in SARS-CoV-2 infection. Furthermore, we found that IL-6, a known cytokine for COVID-19 severity, is also a potential indicator in individuals with diabetes in need of intensive care.

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Duality of Interest. All authors declare that there are no relationships or activities that might bias this study. No potential conflicts of interest relevant to this article were reported.

Author Contributions. I.B.-S., A.F.A.M., T.C.-S., S.N., and H.S contributed to the acquisition of the data or the analysis and interpretation of information. I.B.-S., T.C.-S., S.N., R.L.S., A.B., P.R.S.O., R.K., C.B., M.B.-N., V.B., and N.M.T. contributed to the writing of the manuscript or had substantial involvement in its revision before submission. I.B.-S., S.N., and J.S. conducted the processing of biological samples in the laboratory. I.B.-S., V.B., and N.M.T. were involved in the conception, hypotheses delineation, and design of the study. A.F.A.M., M.R.S.C., and J.R.C. conducted the medical care of the research participants. N.M.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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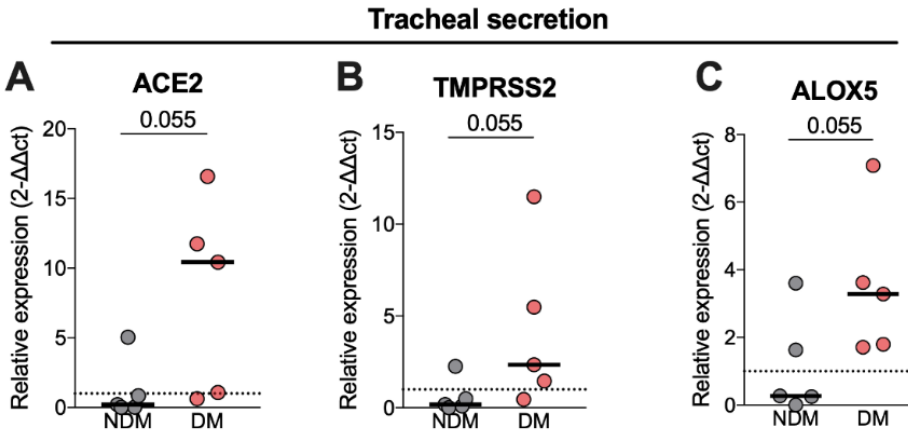
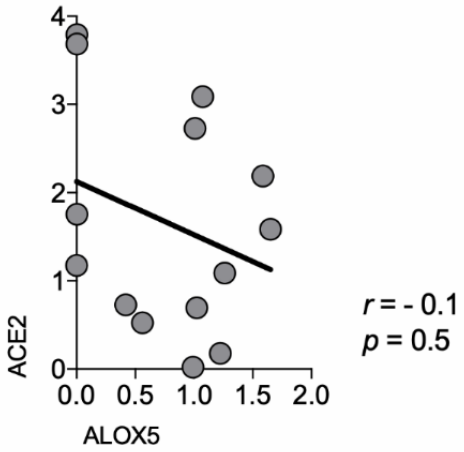
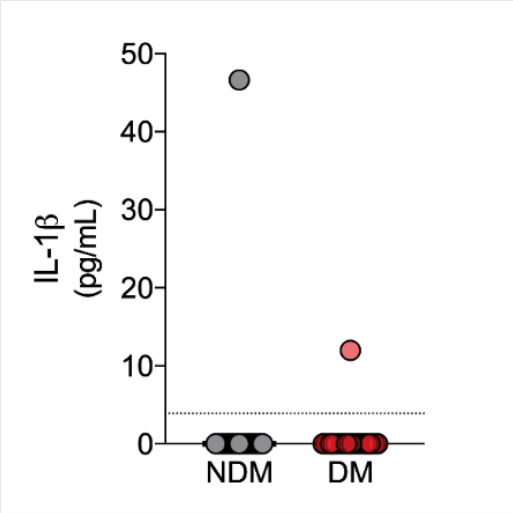
Arquivos suplementares - MANUSCRITO I



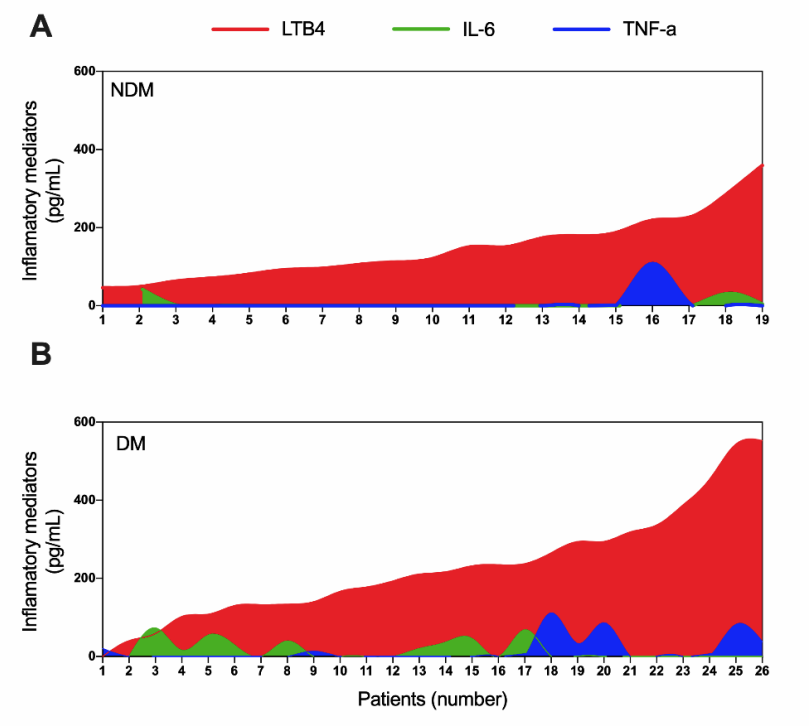
Supplementary Figure 1. Tracheal secretion from COVID-19 patients with DM trends toward increased expression of ALOX5 and ACE2/TMPRSS2 viral receptors. Relative expression of ACE2 (A), TMPRSS2 (B) and ALOX5 (C) in tracheal secretion of NDM and DM patients with COVID-19. Dotted line = control group relarive expression. Data shown as median.



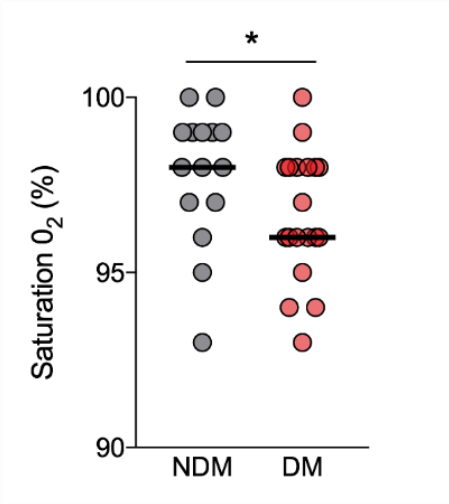
Supplementary Figure 2. No correlation between ACE2 and ALOX5 is observed in NDM COVID-19 patients. Correlation between the expression of ALOX5 and ACE2 in PBMCs of NDM individuals with COVID-19 (A). Spearman r correlation.



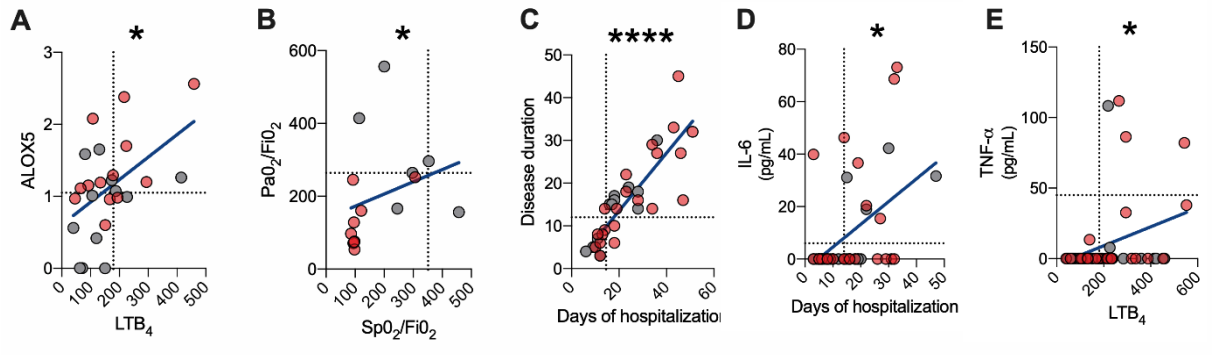
Supplementary Figure 3. No difference in IL-1β production between NDM and DM groups of patients with COVID-19. IL-β serum levels in NDM and DM individuals with COVID-19. Dotted line = cut-off for detection limit. Data shown as median.



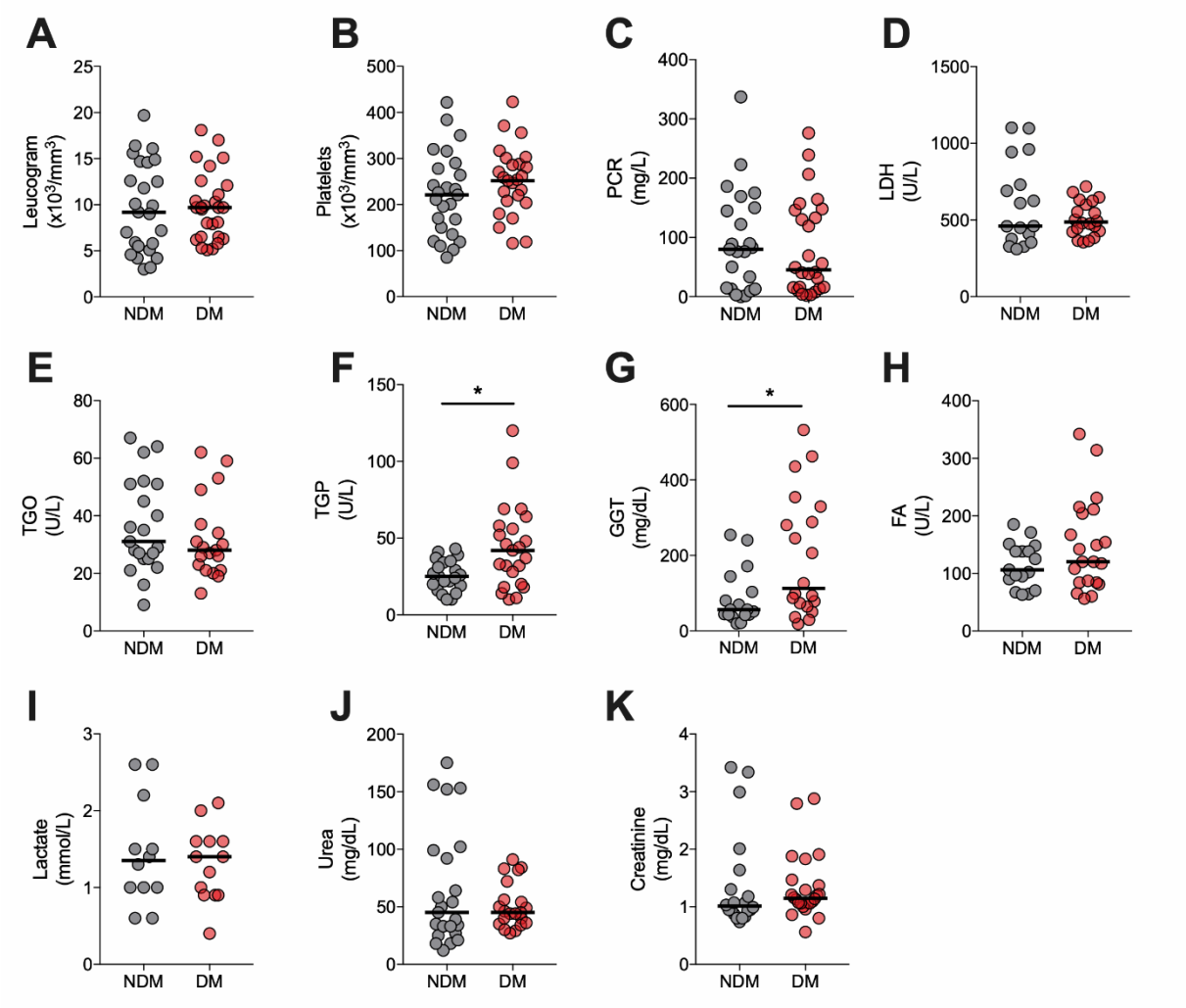
Supplementary Figure 4. LTB_4 is the most prevalent inflammatory mediator at systemic levels. Global and individualized view of LTB4 (red), IL-6 (green) and TNF-α (blue) production in NDM (A) and DM (B) individuals with COVID-19 through Cubic spline analysis.



Supplementary Figure 5. O_2 saturation levels are reduced in DM patients with COVID-19 compared with NDM individuals. SpO2 in NDM and DM patients with COVID-19. Data shown as median. * p<0.05.



Supplementary Figure 6. Correlation between inflammatory mediators and clinical parameters of NDM and DM individuals with COVID-19. Correlation between ALOX5 and LTB_4 (A), PaO_2/FiO_2 and SpO_2/FiO_2 (B), disease duration and hospitalization days (C), IL-6 and hospitalization days (D), TNF-α and LTB_4 (E) in NDM and DM patients with COVID-19. Dotted lines = median of NDM group. Spearman r correlation. * p<0.05. **** p<0.0001.



Supplementary Figure 7. Liver enzymes are altered in DM patients with COVID-19 compared to NDM group. Quantification of Leucogram (A), platelets (B), C-reactive protein (C), Lactate dehydrogenase (D), Glutamic-oxalacetic Transaminase (E), Glutamic Pyruvic Transaminase (F), Gamma-glutamyl transferase (G), Alkaline phosphatase (H), Lactate (I), Urea (J) and Creatinine (K) in NDM and DM patients with COVID-19. Data shown in median. * $p<0.05$.

4 **MANUSCRITO II**

4.1 HIPÓTESE

Inflamação decorrente do risco aumentado para o diabetes (pré-diabetes) é suficiente para agravar a COVID-19.

4.2 OBJETIVOS

4.2.1 **Objetivo geral**

Identificar mecanismos imunológicos observados em indivíduos com pré-diabetes associados ao agravamento da COVID-19.

4.2.2 **Objetivos específicos**

- Caracterizar a população e o estado clínico de pacientes sem diabetes e com pré-diabetes;
- Mensurar os níveis de mediadores inflamatórios no plasma de pacientes com COVID-19 sem diabetes e com pré-diabetes;
- Correlacionar os níveis dos mediadores inflamatórios com a gravidade da COVID-19;
- Analisar as sequelas da COVID-19 após 3 meses do início dos sintomas em pacientes sem diabetes e com pré-diabetes.

4.3 PREDIABETES INDUCES MORE SEVERE ACUTE COVID-19 ASSOCIATED WITH IL-6 PRODUCTION WITHOUT WORSENING LONG-TERM SYMPTOMS

Pré-diabetes induz COVID-19 aguda mais grave associado à produção de IL-6 sem piorar os sintomas a longo prazo

Nesse trabalho, nós buscamos avaliar a influência do pré-diabetes nas fases aguda e de longo prazo da COVID-19. Para isso, comparamos mediadores inflamatórios, parâmetros laboratoriais, clínicos e sintomas em pacientes com COVID-19 com pré-diabetes (PDM) e sem diabetes (NDM) durante a fase aguda e 3 meses após a doença. Os resultados mostram que os pacientes com PDM tiveram maior tempo de internação e necessitaram mais frequentemente de assistência na unidade de terapia intensiva. Na admissão, comparados aos NDM, os pacientes com PDM apresentavam níveis séricos mais elevados de IL-6, mediador que se relacionou com menor saturação e maior gravidade da doença. No pós-COVID-19, o PDM não induziu grandes alterações nos parâmetros laboratoriais e sintomas residuais, mas influenciou o perfil dos sintomas relatados. Assim, este estudo mostrou que o PDM está relacionado ao risco de desenvolvimento de COVID-19 grave e que a IL-6 sérica elevada parece ser um biomarcador promissor de COVID-19 grave nesses pacientes. Além disso, os achados mostram que o PDM, apesar de piorar a COVID-19, não causa sequelas significativas a longo prazo.

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Prediabetes Induces More Severe Acute COVID-19 Associated With IL-6 Production Without Worsening Long-Term Symptoms

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Aims: Pre-existing conditions, such as age, hypertension, obesity, and diabetes, constitute known risk factors for severe COVID-19. However, the impact of prediabetes mellitus (PDM) on COVID-19 severity is less clear. This study aimed to evaluate the influence of PDM in the acute and long-term phases of COVID-19.

Materials and methods: We compared inflammatory mediators, laboratory and clinical parameters and symptoms in COVID-19 patients with prediabetes (PDM) and without diabetes (NDM) during the acute phase of infection and at three months post-hospitalization.

Results: Patients with PDM had longer hospital stays and required intensive care unit admission more frequently than NDM. Upon hospitalization, PDM patients exhibited higher serum levels of interleukin 6 (IL-6), which is related to reduced partial pressure of oxygen (PaO_2) in arterial blood, oxygen saturation (SpO_2) and increased COVID-19 severity. However, at three months after discharge, those with PDM did not exhibit significant alterations in laboratory parameters or residual symptoms; however, PDM was observed to influence the profile of reported symptoms.

Conclusions: PDM seems to be associated with increased risk of severe COVID-19, as well as higher serum levels of IL-6, which may constitute a potential biomarker of severe COVID-19 risk in affected patients. Furthermore, while PDM correlated with more severe acute-phase COVID-19, no long-term worsening of sequelae was observed.

Keywords: prediabetes, inflammation, COVID-19, long COVID, IL-6

INTRODUCTION

The deadly Coronavirus disease 2019 (COVID-19) pandemic due to the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) continues to present an enormous challenge to health systems worldwide. Gaps in our understanding of COVID-19 have undoubtedly exacerbated the death toll of over 6 million people worldwide, according to the World Health Organization.

The impact of the COVID-19 pandemic has been largely accentuated by the transmission capabilities of SARS-CoV-2. This new coronavirus interacts with different host cells by binding its viral SPIKE protein to the host's ACE2 receptor, mediated by proteases such as transmembrane serine protease 2 (TMPRSS2) and FURIN (1).

The spectrum of COVID-19 presentation varies widely, from mild to moderate and severe clinical forms. The severe form of disease occurs predominantly in elderly, hypertensive, obese, and diabetic individuals (2). In the context of diabetes, researchers around the world have been struggling to identify the mechanism underlying increased severe COVID-19 risk in these individuals. Recently, our group demonstrated the involvement of the Leukotriene B4 (LTB_4) pathway in severe cases of COVID-19 in individuals with diabetes, and reported increased expression of ACE2 and TMPRSS2 in peripheral blood mononuclear cells (3). Other studies have also highlighted the importance of increased expression of these SARS-CoV-2 gateway receptors to the pathogenesis of COVID-19 using different cell types placed under hyperglycemic conditions (4–7).

Based on preliminary exploratory study, prediabetes also appears to be a risk factor for severe COVID-19 (8–10). However, the mechanisms that lead to disease exacerbation remain unknown. Moreover, the potential for these patients to develop residual symptoms after acute phase of the COVID-19 has yet to be investigated. Thus, we sought to assess the involvement of inflammatory mediators in PDM individuals with severe COVID-19 requiring hospitalization. Our results indicate that COVID-19 patients with PDM experience a greater degree of lung injury, require prolonged hospitalization and intensive care admission. However, PDM does not seem to impact the long-term prevalence of symptoms post-acute COVID-19. Finally, our results suggest that serum levels of IL-6 may represent a promising marker of unfavorable outcomes associated with PDM in COVID-19 patients.

MATERIALS AND METHODS

Study Approval

This study followed the principles of the Declaration of Helsinki. The Institutional Board for Ethics in Human Research at the Gonçalo Moniz Institute, Oswaldo Cruz Foundation (CAAE 36199820.6.0000.0040), and Irmã Dulce Social Works (CAAE 33366020.5.0000.0047) approved this study. Participants gave informed consent previous to any data and sample collection.

Patients

Patients diagnosed positive for COVID-19, based on the positivity of molecular test (RT-qPCR) or clinical history for COVID-19. In the study of the acute phase of COVID-19, patients were diagnosed through RT-qPCR or clinical plus radiologic criteria and were admitted to Ernesto Simões Filho General and Memorial Hospital, Salvador, Brazil, from July 2020 to February 2021. Forty-two patients were enrolled in this study, 23 without diabetes (NDM) and 19 with prediabetes (PDM). To analyze the consequences of COVID-19, patients with confirmed SARS-CoV-2 infection through RT-PCR/Lateral-flow or serologic tests, three hundred and three patients were enrolled 3 months after symptom onset (acute phase between August 2020 and May 2021) from Octávio Mangabeira Specialized Hospital, Salvador, Brazil, of which 130 are without diabetes and 173 with prediabetes. According to the Brazilian Diabetes Society guidelines, 2019-2020 (Lyra et al., 2020), in this study, patients with HbA1c between ≥ 39 mmol/mol (5.7%) and < 48 mmol/mol (6.5%) were considered with prediabetes (PDM) and patients with values < 5.7% were considered without diabetes (NDM). The score to assess mobility impairment in the post COVID-19 phase was based on EuroQol questionnaire. Clinical data from all patients were obtained on admission from medical records and managed on the REDCap platform. Patients who did not agree to sign the free and informed consent, were pregnant, who did not have the value of glycated hemoglobin, had symptoms for >14 days, and had been in the hospital for >48 h were excluded of this study.

Quantification of Inflammatory Mediators

Blood samples from all patients were collected at admission. Plasma was separated to quantify inflammatory mediators. Based on the highlight of specific inflammatory mediators in the outcome of COVID-19 (Pérez et al., 2021; Tay et al., 2020), serum levels of Tumor Necrosis Factor alpha (TNF-α), Interleukin 6 (IL-6) and LTB_4 (Cayman Chemical, USA) were evaluated using Enzyme Linked Immunosorbent Assay (ELISA).

Gene Expression Analysis

Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) collected at admission using miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's guidelines. Relative expression of ACE2 (assay ID Hs.PT.58.27645939); transmembrane serine protease 2 (TMPRSS2) (assay ID Hs.PT.58.4661363); furin, paired basicamino acid cleaving enzyme (FURIN) (assay ID Hs.PT.58.1294962 were analyzed. cDNA synthesis was performed using the SuperScript III Reverse Transcriptase Kit (Invitro-gen, Carlsbad, CA). Then, cDNA was amplified by quantitative real-time PCR using the SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). Relative gene expression is shown as the fold change between the NDM and PDM groups using b-actin as housekeeping gene (ACTB) (assay ID Hs.PT.39a.22214847). All primers were pur-chased from Integrated DNA Technologies (Coralville, IA).

Statistical Analysis

Data are presented as mean and SD or median and interquartile range values for numerical variables and proportions (%) for categorical variables. For variables with normal distribution, we used Student's t-test (two groups). For non-normal distribution, we used Mann–Whitney test (two groups), Kruskal–Wallis with Dunn's post-test (three or more groups), and the Spearman test we used for correlations analysis. Chi-Square or Fisher's exact test was used to compare proportions. The hierarchical clustering analysis was performed based on the average of the Euclidean distance between symptoms and patients splitted by group using Orange software version 3.28 with patients without missing data. Outliers were identified using ROUT method (Q=1%). All tests were conducted using Prism 8 software (GraphPad, USA). Differences were considered statistically significant when $p < 0.05$, or adj. $p < 0.05$ for multiple comparisons.

RESULTS

High Serum Levels of IL-6 and Severe COVID-19 Outcomes in Prediabetic Patients

This study evaluated 42 patients with COVID-19 in the acute phase of infection: 23 (10 F: 13 M; median age 54 years) non-diabetic (NDM) controls, and 19 prediabetic individuals (06 F: 13 M; median age 67 years). The groups were proportionately similar with regard to comorbidities and symptoms (**Table 1**) and no differences were seen in drug therapy during hospitalization between the groups (see **Supplementary Table 1**).

Our analysis of inflammatory mediators revealed that prediabetic patients demonstrated increased IL-6 levels ($p = 0.0001$) during acute COVID-19; however, no differences were seen in TNF-a or LTB_4 (**Figures 1A-C**). **Figure 1D** shows the percentage of patients who were producers of detectable levels of inflammatory mediators. While both groups produced LTB_4 and none produced TNF-a, approximately 63% of PDM exhibited high levels of IL-6 compared to NDM.

We observed that PDM patients with COVID-19 required extended hospital stays [15 days (IQR 8-22)] compared to NDM [8 days (IQR 5-15)], as illustrated in **Figure 1E** ($p = 0.044$). Among the PDM COVID-19 patients, 78% were admitted to an intensive care unit (ICU), in contrast to 56% of NDM (**Figure 1F**). Additionally, patients with PDM had longer ICU stays than NDM (10 days, IQR 1-17 vs. 2 days, IQR 1-4, respectively, $p = 0.024$) (**Figure 1G**). Regarding mechanical ventilation (MV), 64% of the PDM group required invasive respiratory support, compared to 33% of NDM patients (**Figure 1H**). PDM patients also developed more lung dysfunction based on ratios of oxygen saturation (SpO_2) (mean ± SD: 243.3 ± 143.7 vs 363.8 ± 130.4, $p = 0.009$) and arterial oxygen partial pressure (PaO_2) (185.0 ± 105.9 vs 320.5 ± 171.1, $p = 0.043$) to fractional inspired oxygen (FiO_2) (S/F and P/F ratios, respectively) (**Figures 1I, J**). Together, these findings indicate that PDM is associated with higher IL-6 serum levels and increased risk of severe COVID-19 (lung dysfunction, more frequent and prolonged hospitalization and ICU admission). Our analysis of the expression of gateway receptors for SARS-CoV-2 (ACE2, TMPRSS2 and FURIN) in PBMCs revealed no differences between the groups (see **Supplementary Figure 1**).

IL-6 Serum Levels as a Biomarker of Severe COVID-19 in Patients With Prediabetes

We further sought to identify correlations between IL-6 serum levels and different clinical outcomes as well as laboratory

TABLE 1 | Clinical characteristics among non-diabetic (NDM) and prediabetic (PDM) patients with Coronavirus Disease 2019 (COVID-19) stratified according to low or high IL-6 production.

CHARACTERISTICS	All patients, *n*			PDM patients, *n*		
	NDM (n = 23)	PDM (n = 19)	*p value*	LOW (n = 7)	HIGH (n = 12)	*p value*
Male, n/N (%)	13/23 (56%)	13/18 (72%)	0.300	4/7 (57%)	9/11 (82%)	0.326
Age, mean ± SD	54 ± 19	67 ± 16	0.053	65 ± 17	67 ± 16	0.336
Hb1Ac, median (IQR)	34 (32 - 38)5.3 (5.1 -	42 (39 - 45)6.0 (5.7 -	<0.0001	41 (39 - 44)5.9 (5.7 -	42 (39 - 45)6.0 (5.7 -	0.334
mmol/mol	5.6)	6.3)		6.2)	6.3)	
%						
Onset of symptoms prior to hospitalization (in days), median (IQR), N	7 (5-13)(N=11/23)	4 (2-6)(N=12/19)	0.086	2 (2-4)(N=5/7)	6 (3-13)(N=7/12)	0.093
Comorbidities n/N (%)						
Obesity	0/13 (0%)	4/14 (28%)	0.097	2/5 (40%)	2/9 (22%)	0.580
Hypertension	10/18 (55%)	6/15 (40%)	0.373	3/5 (60%)	3/9 (33%)	0.580
COPD	1/11 (9%)	2/12 (17%)	>0.999	0/3 (0%)	2/9 (22%)	>0.999
Symptoms n/N (%)						
Fever	11/17 (65%)	6/12 (50%)	0.428	2/5 (40%)	4/7 (57%)	>0.999
Cough	14/20 (70%)	12/14 (86%)	0.422	6/6 (100%)	6/8 (75%)	0.472
Dyspnea	12/19 (63%)	15/16 (94%)	0.090	6/6 (100%)	9/10 (90%)	>0.999
Outcome n/N (%)						
Death	3/22 (14%)	6/17 (35%)	0.456	1/6 (16%)	5/11 (45%)	0.333

COPD, Chronic Obstructive Pulmonary Disease; n, Total number of patients; N, Number of patients with information available.

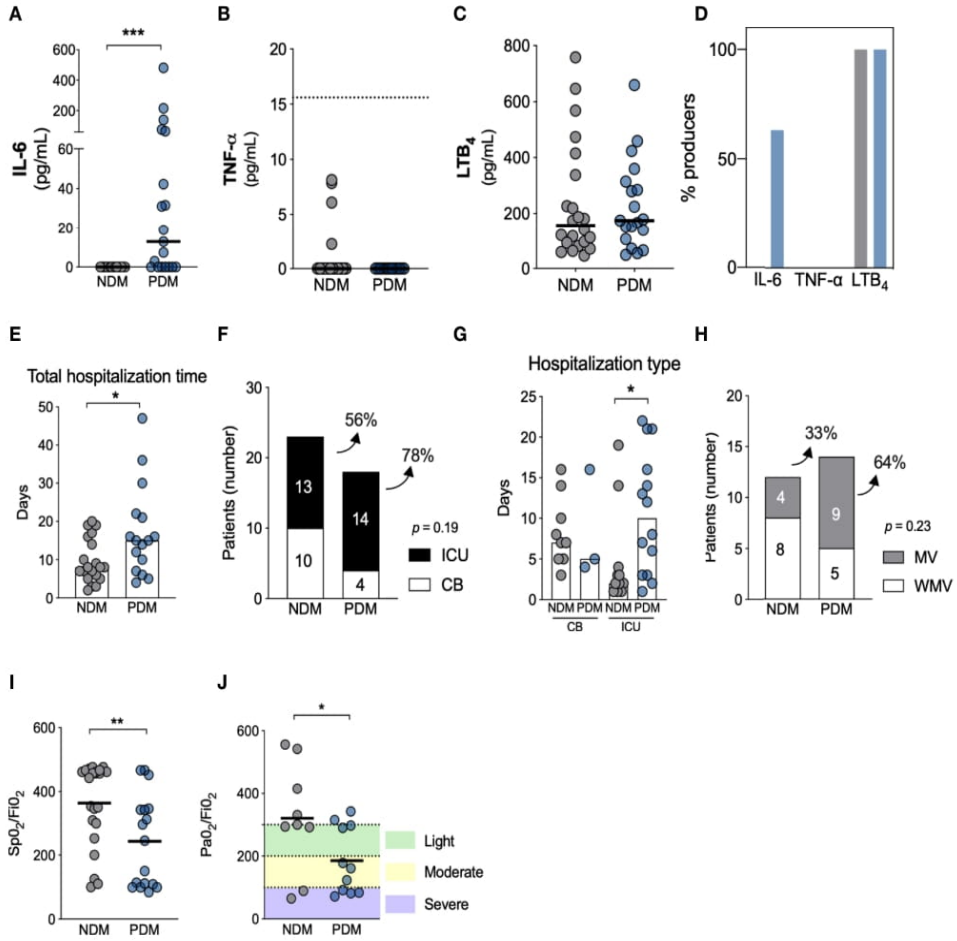

FIGURE 1 | COVID-19 disease is more severe in individuals with prediabetes. Systemic levels of IL-6 **(A)**, TNF-a **(B)** and LTB_4 **(C)** in individuals without diabetes (NDM) and prediabetes (PDM) with COVID-19. **(D)** Percentage of individuals producing inflammatory mediators in NDM and PDM with COVID-19. **(E)** Total hospitalization time between NDM and PDM individuals. **(F)** Number and percentage of NDM and PDM individuals admitted to clinical beds (CB) (white) or intensive care unit (ICU) (black) due to COVID-19. **(G)** Days of hospitalization in ICU or CB in NDM and PDM with COVID-19. **(H)** Number and percentage of patients who required (black) mechanical ventilation or not (white) between NDM and PDM group. **(I)** SpO_2/FiO_2 and **(J)** PaO_2/FiO_2 ratio in NDM and PDM patients with COVID-19. [**(A, B, E)** = Mann Whitney test]; [**(F, H)** = Fisher's exact test]; [**(G)** = Kruskal-Wallis with Dunn's post-test); [**(I, J)** = Unpaired t-test]. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

parameters. Correlation matrixes constructed for NDM (**Figure 2A**) and PDM (**Figure 2B**) patients revealed no associations regarding IL-6 levels in the NDM group (**Figure 2A** – blue bar). However, positive correlations between IL-6 and WBC ($r = 0.5$; $p = 0.057$), lactate dehydrogenase (LDH) ($r = 0.9$; $p = 0.001$), C-reactive protein (CRP) ($r = 0.8$; $p = 0.002$), and urea ($r = 0.6$; $p = 0.014$) were identified in PDM patients. Additionally, IL-6 was observed to negatively correlate with the S/F and P/F ratio ($r = -0.7$; $p = 0.002$ and -0.6; $p = 0.023$, respectively) in PDM (**Figure 2B**).

Within the PDM patients, a subgroup of 12 patients were observed to produce high levels of IL-6 (termed "high producers"), while 7 had undetectable levels of IL-6 (i.e., "low producers"). We found that the high producers of IL-6 presented increased levels of CRP and WBC (**Figures 2C, D**), while lower S/F and P/F ratios were found in high IL-6 producers (**Figures 2E, F**). Unfortunately, laboratory data was not available for all PDM patients who were low and high IL-6 producers.

We further confirmed that higher levels of IL-6 in PDM patients with COVID-19 was associated with ICU admission (**Figure 2G**) and MV (**Figure 2H**). **Figure 2I** indicates that systemic levels of IL-6 >1.4 pg/mL increase the risk of severe COVID-19 in PDM patients with respect to the outcome of ICU admission (AUC = 0.89; sensitivity of 78.5% and specificity of 100%; $p = 0.019$), while >15.9 pg/mL increased the risk of need for MV (AUC = 0.84; sensitivity of 77.8% and specificity of 80%, LR 3.9; $p = 0.038$) (**Figure 2I**). Finally, high serum levels of IL-6 were also observed in some PDM patients who died following ICU admission (**Figure 2J**). These findings suggest that serum levels of IL-6 are associated with COVID-19 severity in PDM individuals (see **Supplementary Table 2**).

Prediabetes Does not Worsen Complications in Long-Term COVID-19

We investigated the impact of PDM at 3 months after acute COVID-19 by analyzing laboratory parameters, quality of

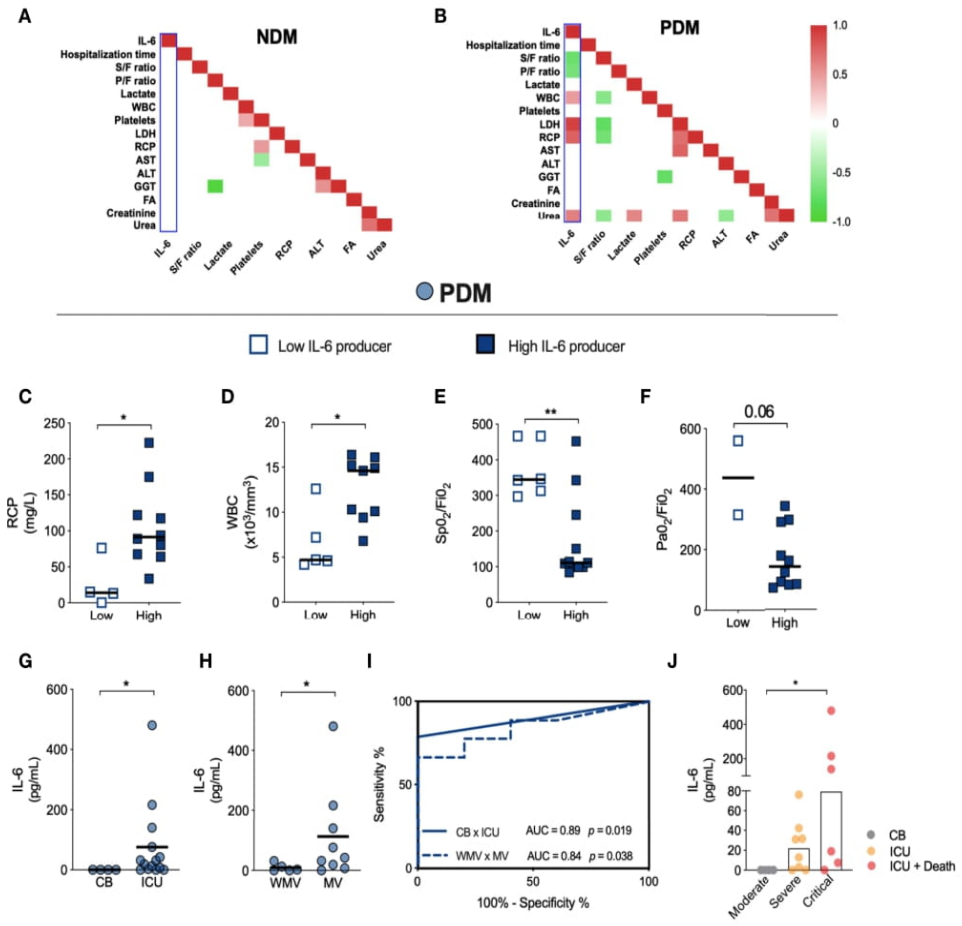

FIGURE 2 | IL-6 production induced by prediabetes dictates the severity of COVID-19. Correlation matrix between clinical parameters of NDM **(A)** and PDM **(B)** patients with COVID-19. Values of **(C)** PCR, **(D)** WBC, **(E)** S/F and **(F)** P/F ratio in PDM patients with low (empty blue box) or high (full blue box) production of IL-6 cytokine. **(G)** IL-6 levels produced by PDM patients hospitalized in CB or ICU. **(H)** IL-6 levels produced by PDM patients who required (MV) or not of ventilation mechanic (WMV). **(I)** Analysis of ROC curve based on IL-6 production in PDM patients in ICU hospitalization (full line) and need for invasive mechanical ventilation (dotted line). **(J)** IL-6 production induced by prediabetes in patients in the moderate, severe and critical form of COVID-19. Correlation positive (red), correlation negative (green). [**(A, B)** = Spearman]; [**(C–H)** = Mann Whitney test]; [**(J)** = Kruskal-Wallis with Dunn's post-test]. *$p < 0.05$; **$p < 0.01$.

life and residual symptoms in 130 NDM and 170 PDM patients with post-acute symptoms of COVID-19 (PASC). Patients were matched for age, sex, comorbidities, and disease severity according to ICU admission (**Table 2**). With the exceptions of increased ALT and Urea in PDM patients, no significant differences were noted in the other laboratory parameters analyzed three months following the acute phase of COVID-19 (**Figure 3A**). Despite higher ALT (median of 26 U/L vs 21 U/L, p = 0.0185) and Urea (median of 30.0 mg/dL vs 26.5 mg/dL, p = 0.0057) levels, values remained within respective reference ranges (See **Supplementary Figure 2**).

Based on EuroQol questionnaire results, with scores ranging from 1 (no impairment) to 5 (extremely severe impairment), only 1% of PDM patients reported a score in the mobility dimension (**Figure 3B**). With respect to the other domains, no significant differences were observed (data not shown).

Regarding residual symptoms reported at three months after disease onset, dyspnea (~50%) and fatigue (~43%) were the most frequent, yet no differences among these symptoms were seen between the NDM and PDM patients (**Figure 3C**). For further assessment, an unsupervised analysis was performed to identify hierarchical clustering of symptoms. After removing missing data, 167 patients were analyzed (67 NDM and 100 PDM). Two well-defined clusters were evidenced in both NDM and PDM patients, an oligosymptomatic cluster 1 (green) and a polysymptomatic cluster 2 (red) (**Figure 3D**). The groups were proportionately similar in terms of age, gender, ICU admission and need for invasive mechanical ventilation (IMV) (**Supplementary Table 3**).

In patients with more post-acute COVID-19 symptoms (polysymptomatic), with the exception of cough [30% (PDM) vs 70% (NDM), odds ratio (OR), 0.17, 95% confidence interval (CI) 0.05 to 0.5, p = 0.0028], PDM patients more frequently reported body pain (78% vs 37%, OR 5.9, 95% CI 1.82 to 17.81, p = 0.0025), anosmia (30% vs 0%, OR infinity, 95% CI 1.9 to infinity, p = 0.0100) and ageusia (26% vs 0%, OR infinity, 95% CI 2.543 to infinity, p = 0.0043) compared to NDM patients

TABLE 2 | Clinical characteristics between individuals without diabetes (NDM) and with prediabetes (PDM) after 3 moths of the COVID-19 acute phase.

CHARACTERISTICS	Patients, *n*		
	NDM (n = 130)	**PDM (n = 173)**	***p value***
Male, (%)	56 (43%)	83 (48%)	0.467
Age, mean ± SD	53 ± 12	54 ± 11	0.259
Hb1Ac %, (Min-Max)	5.3 (3.6 - 5.6)	5.9 (5.7 - 6.4)	<0.0001
Comorbidities n/N (%)			
Obesity	20/65 (31%)	46/104 (44%)	0.081
Hypertension	46/125 (37%)	67/161 (42%)	0.408
COPD	5/124 (4%)	5/160 (2%)	0.510
Post-COVID symptoms n/N (%)			
Dyspnea	35/68 (57%)	51/104 (49%)	0.755
Fatigue	31/68 (45%)	43/104 (41%)	0.582
Headache	21/68 (31%)	29/104 (28%)	0.672
Chest pain	21/68 (31%)	28/103 (27%)	0.600
Cough	20/68 (29%)	25/100 (24%)	0.412
Body pain	19/68 (28%)	30/104 (29%)	0.897
Anosmia	5/68 (7%)	10/104 (10%)	0.607
Ageusia	3/68 (4%)	9/104 (9%)	0.368
Anorexia	3/67 (4%)	8/104 (8%)	0.530
Dysphonia	0/68 (0%)	2/103 (2%)	0.518
Dysphagia	0/68 (0%)	2/103 (2%)	0.518
Severity n/N (%)			
Admission to the ICU	32/62 (52%)	57/127 (45%)	0.384

COPD, Chronic Obstructive Pulmonary Disease; ICU, Intensive Care Unit; n, Total number of patients; N, Number of patients with information available.

(**Figure 3E**). On the other hand, regarding patients with fewer reported symptoms post-acute COVID-19 (oligosymptomatic), PDM patients reported more coughing (19% vs 2%, OR 9.2, 95% CI 1.60 to 100.5, $p = 0.0125$) and less olfactory dysfunction (1% vs 12%, OR 0.09, 95% CI 0.008 to 0.77, $p = 0.0116$) than NDM (**Figure 3F**).

These findings reveal that despite the presence of post-COVID-19-related symptoms in both groups, differences in the profiles of reported symptoms were evidenced between the polysymptomatic and oligosymptomatic patients. Oligosymptomatic PDM patients present more cough and less anosmia, while more body pain, anosmia, ageusia and less cough were reported by polysymptomatic PDM compared to their respective NDM counterparts. Importantly, no differences in the profile of post-COVID-19-related symptoms were observed in patients admitted to the ICU or those requiring IMV during the acute phase of disease compared to those with milder COVID-19, suggesting that post-acute COVID-19 symptomatology was not dependent on disease severity in the studied patients.

DISCUSSION

Isolated studies have reported that prediabetes implies an increased risk of severe infection and mortality by COVID-19 (10–12); however, the pathogenic mechanism underlying this risk remains unclear. It was recently demonstrated that 6% to 39.4% of individuals with PDM develop severe COVID-19 (9–11, 13, 14). Although these studies are still scarce, there is accumulating evidence that prediabetes, as well as diabetes, may also culminate in severe COVID-19 (8, 10, 15). The findings reported herein suggest that IL-6 may play a role in the increased risk of severe COVID-19 in PDM individuals. However, it is important to consider that our study was limited in terms of its sample size. PDM patients in the acute phase of infection were older (67 vs 54 years) and more obese (28% vs 0%) than NDM individuals, despite a lack of statistical significance. However, the PDM individuals herein were found to have significantly higher rates of ICU admission and IMV than the NDM patients, which is concordant with age and obesity being known risk factors for severe COVID-19 (16–18).

Previous studies have demonstrated the influence of hyperglycemia on the expression patterns of SARS-CoV-2 gateway receptors, such as ACE2 and TMPRSS2 (3, 4). In this context, differently than COVID-19 patients with diabetes who exhibited increased ACE2 and TMPRSS2 receptor expression in PBMCs, the PDM patients investigated herein had no altered expression patterns for these receptors (3). On the other hand, as with individuals with diabetes, the extent of lung injury (based on the P/F and S/F ratio) was also observed to be significantly greater in patients with prediabetes (3).

Previous studies have reported the impact of hyperglycemia on severe outcomes of COVID-19 in the acute phase, as well the reduced efficiency of tocilizumab therapy, an inhibitor of IL-6 cytokine signaling (19, 20). An exacerbated inflammatory state is known to be one of the main triggers for severe COVID-19 (1). Associations between IL-6 production and COVID-19 severity have been widely reported (1, 3, 21). While the production of this mediator appears to be related to blood glucose levels (22) in the context of COVID-19, no associations with prediabetes have

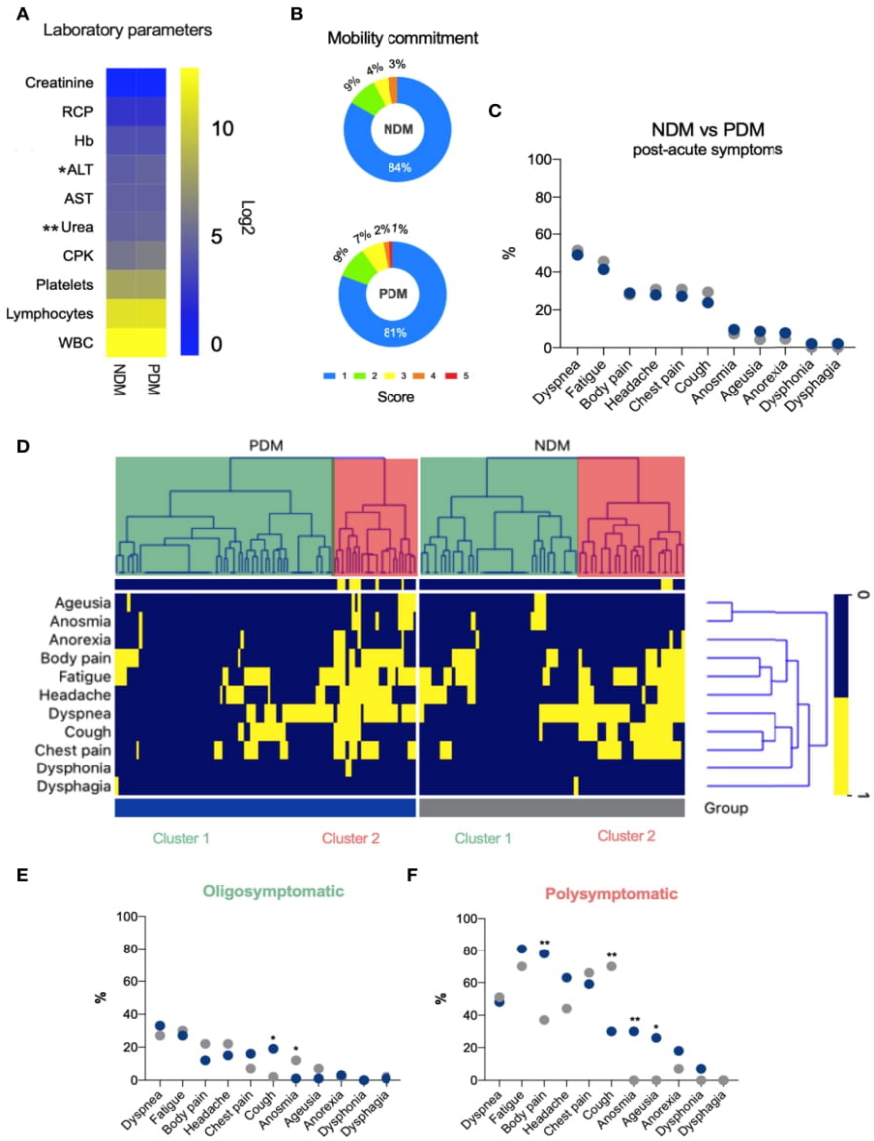

FIGURE 3 | Prediabetes does not alter the symptoms of long-term COVID-19. **(A)** HeatMap shown the median of laboratory parameters values in NDM and PDM patients 3 moths after COVID-19 (blue = low values; yellow = high values). **(B)** Percentage of the degree of mobility impairment in NDM and PDM patients. **(C)** Percentage of symptoms in NDM and PDM patients after COVID-19. **(D)** HeatMap clustered showing symptoms reported by NDM and PDM patients 3 months after COVID-19 (Blue = negative and Yellow = positive for symptoms). **(E)** Percentage of symptoms in NDM and PDM in a population of patients with the highest Percentage of symptoms in NDM and PDM in a population of patients with the highest **(E, F)** percentage of symptoms after COVID-19. (A = Mann Whitney test); (E, F = c^2 test or Fisher's exact test). *$p < 0.05$; **$p < 0.01$

been reported to date (1, 21, 23). Our findings indicate that similarly to diabetic patients, those with prediabetes also induce increased levels of IL-6 during the acute phase of COVID-19 (3, 22). However, while other inflammatory mediators, such as Leukotriene B4, were found to be elevated in patients with diabetes and COVID-19, this was not the case in the PDM patients studied herein (3). As approximately 63% of the PDM patients evaluated produced high levels of IL-6, it is possible that this inflammatory mediator may be a relevant factor in driving patients with prediabetes to develop severe COVID-19 (9–11, 13, 14).

Elevated IL-6 production by individuals with prediabetes was found to correlate with important parameters of severe COVID-19, such as LDH, CRP, and low O_2 saturation. Koh et al. showed that CRP was a key biomarker associated with diabetes-induced severe COVID-19 (14). In addition, the relationship between IL-

6 production and increased CRP has also been reported as a possible trigger for severe COVID-19 (21). LDH has additionally been cited in cases with severe COVID-19, as well as a participation in the activation of inflammasomes, which is related to a worse prognosis of the disease (24, 25). These findings reinforce the notion that enhanced IL-6 production may indeed be associated with a worse prognosis of COVID-19, as the PDM patients evaluated herein produced higher levels of this cytokine non-diabetic COVID-19 patients and were more likely to experience severe outcomes.

Although serious complications have been associated with the acute phase of COVID-19, disease recovery can occur slowly in some cases and may imply residual symptoms, termed "long COVID." (26, 27) Consistent with our results, Fernández-de-las-Peñas et al. showed that the symptoms reported by patients with diabetes were not different from those without diabetes (28). The present findings indicate that the most frequently reported symptoms in our patients with long COVID-19 were dyspnea, fatigue, headache, cough and body pain, which is consistent with the symptomatology reported in other studies (26, 27, 29).

The persistence of residual symptoms post-COVID-19 requires further study. Importantly, patient sex may be a factor for the reporting of these symptoms regardless of glycemic level (as evidenced by the predominance of females in the polysymptomatic group). Previous reports have argued that sociocultural aspects may be relevant, as women tend to be more concerned with their health (29–31).

In conclusion, despite a relatively limited sample size in the acute phase of SARS-CoV-2 infection, our results indicate that individuals with prediabetes faced an increased risk of developing severe COVID-19, which correlated with high serum levels of IL-6 in these patients. Furthermore, while prediabetes was not shown to significantly exacerbate symptomatology post-acute COVID-19, prediabetic patients present different symptom profiles, depending on their oligosymptomatic and polysymptomatic status, compared to non-diabetic individuals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Board for Ethics in Human Research at the Gonçalo Moniz Institute, Oswaldo Cruz Foundation (CAAE 36199820.6.0000.0040), and Irmã Dulce Social Works (CAAE 33366020.5.0000.0047). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IB-S, TC-S, SN, RK, PO, AB, CS, CB, MB-N, VB, and NT contributed to the article's writing or substantial involvement in its revision before submission. MC, AM, and JC conducted the medical care of the research participants. IB-S, JS, SN, AI and LE processed the biological samples and performed the laboratory essays. IB-S, AM, contributed to the acquisition of the data or the analysis and interpretation of information. IB-S, NT, and VB were involved in the study's conception, hypotheses delineation, and design. NT is this work's guarantor. She had full access to all the study's data and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.896378/full#supplementary-material

Supplementary Figure 1 | Expression of gateway receptors for SARS-CoV-2 is not altered in PBMCs from patients with prediabetes and COVID-19. Gene expression of **(A)** ACE2, **(B)** TMPRRS2 and **(C)** FURIN in peripheral blood mononuclear cells (PBMCs) from patients with COVID-19, without diabetes or with prediabetes.

Supplementary Figure 2 | Laboratory parameters after 3 months of the acute phase of COVID-19. Values of **(A)** Urea, **(B)** Alanine aminotransferase, **(C)** Aspartate aminotransferase, **(D)** Creatinofosfoquinase, **(E)** Hemoglobin, **(F)** Cretinine, **(G)** Platelets, **(H)** C-reactive protein, **(I)** White Blood Cells and **(J)** Lymphocytes in NDM and PDM patients 3 moths after COVID-19. Gray region = limit of reference values. Mann Whitney test, $^{*}p < 0.05$; $^{**}p < 0.01$.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Arquivos suplementares - MANUSCRITO II

Table S1. Medications used during hospitalization for COVID-19 in patients without diabetes (NDM) and with prediabetes (PDM).

	Patients, *n*		
CHARACTERISTICS	**NDM (n = 23)**	**PDM (n = 19)**	*p value*
Systemic corticosteroid n, N (%)	13/22 (59%)	13/16 (81%)	0.178
Anticoagulant n, N (%)	16/20 (80%)	13/14 (93%)	0.378
Dobutamine n, N (%)	0/22 (0%)	3/17 (18%)	0.074
Noradrenaline n, N (%)	5/22 (23%)	9/17 (53%)	0.091
Antiviral n, N (%)	5/21 (24%)	7/15 (47%)	0.175

n = Positive numbers; N = Number available

Table S2. Multivariate logistic regression analysis for severe COVID-19 outcome.

Characteristic	**OR[1]**	**95% CI[1]**	**p-value**
Sex			
Female	—	—	
Male	0.86	0.16, 4.35	0.9
Age (years)	0.98	0.94, 1.02	0.4
Condition			
NDM	—	—	
PDM	2.27	0.38, 15.3	0.4
IL-6 Producer (yes)	19.1	2.71, 401	0.012

Table S3. Sociodemographic characteristics of oligosymptomatic and polysymptomatic patients with symptoms after 3 months of COVID-19 acute phase.

	Oligosymptomatic			Polysymptomatic		
CHARACTERISTICS	**NDM (n = 40)**	**PDM (n = 73)**	*p value*	**NDM (n = 27)**	**PDM (n = 27)**	*p value*
Male, n/N (%)	25/40 (62%)	41/73 (56%)	0.513	7/27 (26%)	7/27 (26%)	>0.999
Age, mean ± SD	53 ± 10	56 ± 12	0.222	53 ± 10	53 ± 10	0.813
Admission to ICU n, N (%)	14/22 (64%)	27/61 (44%)	0.119	6/13 (46%)	7/18 (37%)	0.598
IMV required n, N (%)	5/14 (36%)	6/27 (22%)	0.462	3/6 (50%)	2/7 (28%)	0.592

ICU = Intensive Care Unit; IMV = Invasive Mechanical Ventilation; n = Total number of patients; N = Number of patients with information available.

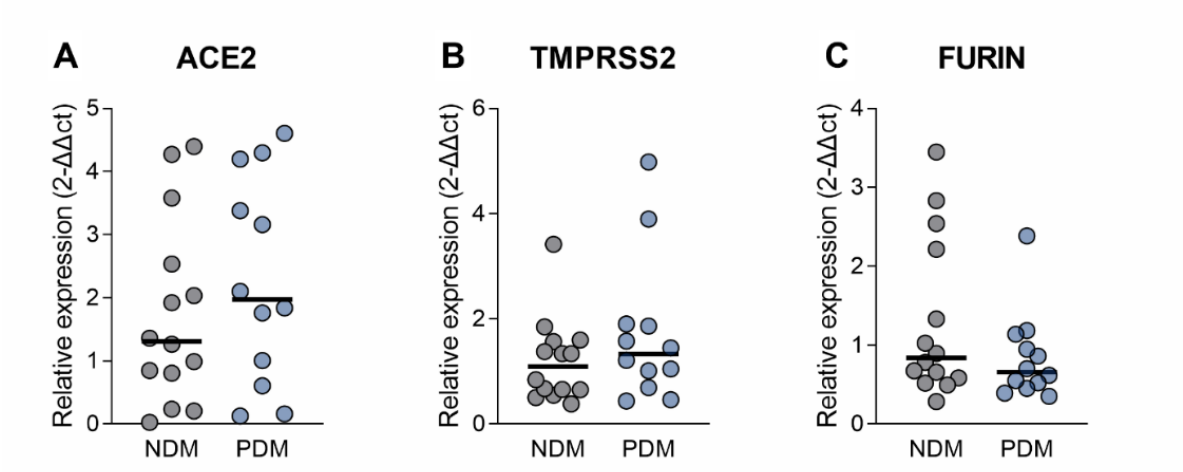

Figure S1. Expression of gateway receptors for SARS-CoV-2 is not altered in PBMCs from patients with prediabetes and COVID-19. Gene expression of (A) ACE2, (B) TMPRRS2 and (C) FURIN in peripheral blood mononuclear cells (PBMCs) from patients with COVID-19, without diabetes or with prediabetes.

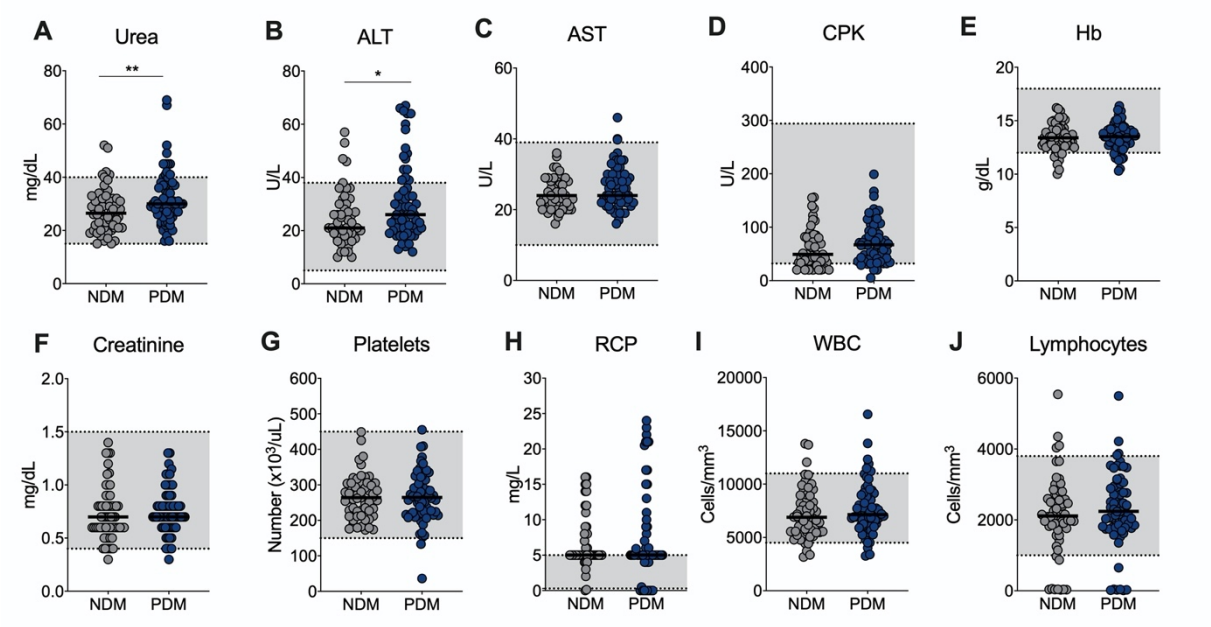

Figure S2. Laboratory parameters after 3 months of the acute phase of COVID-19. Values of (A) Urea, (B) Alanine aminotransferase, (C) Aspartate aminotransferase, (D) Creatinofosfoquinase, (E) Hemoglobin, (F) Cretinine, (G) Platelets, (H) C-reactive protein, (I) White Blood Cells and (J) Lymphocytes in NDM and PDM patients 3 moths after COVID-19. Gray region = limit of reference values. Mann Whitney test, * $p < 0.05$; ** $p < 0.01$.

5 **MANUSCRITO III**

5.1 HIPÓTESE

Marcadores laboratoriais de rotina servem como indicadores dos níveis de produção de IL-6 durante a COVID-19.

5.2 OBJETIVOS

5.2.1 **Objetivo geral**

Identificar e caracterizar subpopulações de produtores de IL-6 durante a COVID-19 associadas à gravidade da doença.

5.2.2 **Objetivos específicos**

- Identificar e caracterizar subpopulações de pacientes com COVID-19 de acordo com os níveis de produção de IL-6;
- Associar subpopulações de produtores de IL-6 com a gravidade da COVID-19;
- Identificar biomarcadores laboratoriais capazes de definir as subpopulações de produtores de IL-6 na COVID-19.

5.3 CLINICAL CHARACTERIZATION AND LABORATORY SIGNATURE OF DIFFERENT IL-6 PRODUCERS IN COVID-19

Caracterização clínica e assinatura laboratorial de diferentes produtores de IL-6 na COVID-19

Diante da importância do mediador IL-6 na gravidade da COVID-19 e da dificuldade de identificar pacientes alto produtores em exames de rotina, nesse estudo nós buscamos caracterizar e identificar subpopulações de produtores de IL-6 na fase aguda da COVID-19. Para isso, nós mensuramos os níveis de IL-6 no plasma de 405 pacientes com COVID-19 e identificamos as subpopulações por meio da produção diferenciada de IL-6. Em seguida, caracterizamos, associamos com desfechos clínicos e avaliamos possíveis marcadores laboratoriais capazes de predizer as subpopulações de produtores de IL-6 na COVID-19. Nós identificamos 3 subpopulações com base na formação de clusters (não, baixo e alto produtor de IL-6). Os altos produtores de IL-6 necessitaram de maiores cuidados intensivos, ventilação mecânica invasiva e uso de corticoides, além de possuírem um tempo de sobrevivência menor decorrente da COVID-19. Nós também verificamos que marcadores laboratoriais de rotina podem ajudar identificar essas subpopulações de produtores de IL-6, sendo a proteína C reativa, a de maior destaque. Assim, nesse estudo foi possível observar que existe uma relação entre o nível de produção de IL-6 e diferentes desfechos na COVID-19, sendo que os altos produtores são os com piores prognósticos. Além disso, exames laboratoriais de rotinha podem auxiliar na identificação de diferentes produtores de IL-6.

CLINICAL CHARACTERIZATION AND LABORATORY SIGNATURE OF DIFFERENT IL-6 PRODUCERS IN COVID-19

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Word count: 3.692 words

Abstract

Aims: COVID-19 pandemic has left its mark all over the world, with millions of deaths resulting from the disease. Nowadays, it is known that the severe form of COVID-19 is associated with an exacerbated inflammation, named cytokine storm. Interleukin 6 (IL-6) is a key cytokine is this process, it is known that in the development of COVID-19 there are no, low and high producers for this mediator, however, it is not measured routinely in public hospitals. This limitation renders the assessment of IL-6 production underused as a potential indicator of disease progression. The aim of this study is to characterize and to identify, through routine exams, high and low IL6 producers. **Materials and methods**: Blood samples from 405 patients with COVID-19 were collected on hospital admission. Plasma samples were harvested and assessed for IL-6 production. The levels of IL-6 defined subsets of patients based on high or low producers. After, the groups were clinically characterized and routine laboratory tests were searched as group indicative. **Results**: We found that high IL-6 producers require more intensive care assistance, mechanical ventilation, use of steroids and have a shorter survival

time. Leukocytes, platelets, lactate, C-reactive protein (CRP), lactate dehydrogenase, creatine kinase isoform B (CKMB), creatinine and urea levels were able to distinguish high and low IL-6 producers. However, CRP is the variable most associated with IL-6 production in COVID-19. **Conclusions**: The results highlight the potential of IL-6 as a predictor of disease severity in COVID-19. In addition, different routine laboratory tests can be used to identify high and low producers of IL-6, especially CRP.

Introduction

Scientific knowledge about Coronavirus disease 2019 (COVID-19) has been continuously and rapidly advancing. However, the consequences of COVID-19 pandemic still impact the world population and health services. With more than six million deaths around the world, this disease caused by the infection of the new coronavirus SARS-CoV-2 is a major public health and economical problem (WHO).

It is known that SARS-CoV-2 infection occurs through the interaction of its SPIKE protein with gateway receptors such as the angiotensin 2 converting enzyme (ACE2) in the host. After virus internalization mediated by proteins such as serine 2 transmembrane protease (TMPRSS2) and FURIN, a cytokine storm is released, causing tissue damage and loss of function, mainly in lungs (Moore & June, 2020; Tay et al., 2020). Among the different inflammatory mediators released, interleukin 6 (IL-6) has been widely reported as a key mediator of severe COVID-19, which may require intensive care assistance, mechanical ventilation and culminate in death (Bonyek-Silva et al., 2021; Broman et al., 2021; Jones & Hunter, 2021; Moore & June, 2020; Tay et al., 2020; Y. Z. Zhou et al., 2021). It is known that high levels of IL-6 are related to the immunopathology of respiratory failure in the COVID-19 (Gubernatorova et al., 2020). These levels of IL-6 in patients with COVID-19, in addition to being increased, are heterogeneous among patients, thus, different from previous studies, where IL-6 levels are quantified after stratification by disease severity, in this study, we seek to understand the impact of low and high IL-6 producers prospectively.

IL-6 is a cytokine that participates in innate immunity with pleiotropic functions. According to receptor recognition, IL-6 may act as an anti-inflammatory (binding with membrane receptor) or pro-inflammatory (binding with soluble receptor) mediator (Honore et al., 2020; Patra et al., 2020). In severe COVID-19, IL-6 inhibitors have been used as an alternative of therapy for disease control, such as Tocilizumab. The use of Tocilizumab has

been strategically indicated for some patients, considering the severity and time of the disease (Honore et al., 2020; Matthay & Luetkemeyer, 2021; Wu et al., 2021).

Due to the importance of IL-6 in the inflammatory response, it is crucial to identify subsets of high producer patients at the earliest. Together with the limitation of routinely measure IL-6 in hospitals with low resources, mainly in developing countries, the aim of this study is to evaluate whether routine laboratory markers can identify different IL-6 producers. Thus, the results presented herein should contribute to an appropriated use of IL-6 inhibitor drugs, which are already scarce in the health system of these countries.

Materials and methods

Study approval

This study followed the principles of the Declaration of Helsinki. The Institutional Board for Ethics in Human Research at the Gonçalo Moniz Institute, Oswaldo Cruz Foundation (CAAE 36199820.6.0000.0040), and Irmã Dulce Social Works (CAAE 33366020.5.0000.0047) approved this study. Participants gave informed consent previous to any data and sample collection.

Patients

For this study, diagnosed patients were considered those who had a positivity of molecular test (RT-qPCR) or clinical history for COVID-19. The patients were admitted in Ernesto Simões Filho General and Memorial Hospital, Salvador, Brazil. Four hundred and five patients were recruited from the period from August of 2020 to February of 2021. Clinical data from all patients were obtained on admission from medical records and managed on the REDCap platform. The different reference values between men and women, when analyzed in groups, were considered to have the highest upper and lower limits. Patients who did not agree to sign the free and informed consent, were pregnant, had symptoms for >14 days, and had been in the hospital for >48 h were excluded of this study. The study cohort was matched for time of symptom onset (see table 1).

Quantification of IL-6 levels and group definition

Blood samples from all patients (n = 405) were collected at admission. Plasma was separated to quantify the IL-6 levels using Enzyme Linked Immunosorbent Assay (ELISA)

(R&D Systems, USA). Non-producer (n = 127), low (n = 215) and high (n = 63) IL-6 producer groups were defined based on hierarchical clustering using an unsupervised analysis.

Statistical analysis

Data are presented as mean and SD or median and interquartile range values for numerical variables and proportions (%) for categorical variables. For variables with normal distribution, we used ANOVA (three or more groups). For non-normal distribution, we used Mann–Whitney test (two groups), Kruskal–Wallis with Dunn's post-test (three or more groups), and the Spearman test we used for correlations analysis. Chi-Square or Fisher's exact test was used to compare proportions. The hierarchical clustering and decision tree analysis was performed using Orange software version 3.28. Outliers were identified using ROUT method (Q=1%). The tests were conducted using Prism 8 software (GraphPad, USA). Differences were considered statistically significant when $p < 0.05$, or adj. $p < 0.05$ for multiple comparisons.

Results

Subsets of IL-6 producers during COVID-19

It has been reported that IL-6 is a potential marker for severity in COVID-19. However, patients produce different levels of IL-6, from none to low and high producers. Then, we assessed IL-6 production by patients with different outcomes of COVID-19. Based on the ROC curve (Figure 1A), IL-6 is a potential indicator of hospitalization type (>11.85pg/mL; AUC= 0.72; sensitivity of 71.3% and specificity of 66.6%, LR 2.1; p= <0.0001), need of mechanical ventilation (>18.28pg/mL; AUC= 0.73; sensitivity of 74.3% and specificity of 63.2%, LR 2.0; p= <0.0001) and death (>23.20pg/mL; AUC= 0.77; sensitivity of 72.9% and specificity of 65.0%, LR 2.0; p= < 0.0001) due to complications of COVID-19 (Figure 1A).

In order to identify subsets of IL-6 producers, an unsupervised hierarchical clustering analysis was carried out with 405 COVID-19 patients. Four clusters of patients were identified based on the level of IL-6 production (Figure 1B) and named as high, low or non-producers. Patients with levels of IL-6 above 319.8pg/ml (IQR 174.6 - 490.1) were considered high producers (Clusters 2-4), while low producers (Cluster 1) have 26.3pg/mL (IQR 15.7 - 51.3) on average and non-producers (NP have undetectable levels of IL-6.

In view of the statistical analysis, clusters 2-4 are not different from each other but are statistically different from clusters 1 and NP (p = < 0.0001) (Figure 1C). Figure 1D shows that high IL-6 producers predominantly develop severe COVID-19 compared to those with the

moderate form (18.4% vs 1.6%). These findings indicate that the subset of patients with high IL-6 levels should be further explored and early identified during admission to health centers.

Regarding group pairing, it is important to mention that there is no difference between sex and comorbidities, with the exception of heart disease. On the other hand, the median age of IL-6 producers is relatively higher compared to NP. Interestingly, in terms of symptoms, the low and high IL-6 producers reported less dyspnea and myalgia compared to NP patients (see Table 1).

IL-6 levels dictate different outcomes of COVID-19

Next, we sought to investigate whether IL-6 production is associated with different outcomes of COVID-19. The results show that almost all high IL-6 producers (98%) require intensive care assistance (ICU), a significant high percentage compared to low (89%) and NP (69%). This is also similar for the need of mechanical ventilation (MV), where 76% of high IL-6 producers required MV, a percentage 7.6 times higher than NP (10%) and 1.8 times, compared to low producers (42%). In addition, high IL-6 producers also required corticosteroid therapy more frequently (91%) than NP (67%) and low producers (81%). Finally, we assessed the final outcome of COVID-19 (hospital discharge or death) and the results are alarming, where 67% of high IL-6 producers died due to complications from COVID-19, compared with 5.3% and 31% of NP and low producers, respectively (Figure 2A). These findings highlight that early identification of patients with high levels of IL-6 could improve their therapy and reverse this prognosis.

We also investigated whether IL-6 production could be associated with the length of hospital stay. Regarding hospitalization in clinical beds (BC), there is no difference between IL-6 producers. However, for those admitted in ICU, high IL-6 producers spend 20 days (IQR 16-39) from symptoms onset to hospital discharge, compared to 18 days for low (IQR 15-22) and 14 days for NP patients (IQR 10-19). Regardless of hospitalization type, high IL-6 producers require prolonged hospital stay (Figure 2B).

Lungs are frequently affected in severe COVID-19 and high IL-6 producers develop significantly more lung injury based on SpO_2/FiO_2 and PaO_2/FiO_2 ratios. High IL-6 producers had a median SpO_2/FiO_2 ratio of 163 (IQR 112-283) compared to 237 of low (IQR 107 - 348) and 254 of NP (IQR 118-456). Comparing lung injury measured by PaO_2/FiO_2 ratio, high IL-6 producers have a median of 184 (IQR 116-261) compared to 188 of low (IQR 106 - 338) and

266 of NP (IQR 129 - 412), which is considered as light lung injury in 61%, 32% and 40% for NP, Low and High IL-6 producers, respectively (Figure 2C).

The major outcome of COVID-19 is patient survival or death, in this context, high IL-6 producers have a shorter life span, with a median of 7 days (IQR 2-14), compared to 14 days of NP (IQR 5-32) and 12 days of low producers (IQR 5-20) (Figure 2D). Figure 2E shows the probability of death for each group, where high IL-6 producers have a shorter mean of survival, 14 days on average, compared to 37 days of NP and 23 days of low producers.

Laboratory parameters are altered in IL-6 producers during COVID-19

It has already been demonstrated the importance of identify IL-6 producers early during COVID-19. However, its quantification requires time, investment, and skilled labor, which render it unfeasible in most public hospitals, especially in developing countries. Thus, we sought to identify routine laboratory parameters capable of predict high IL-6 producers.

Markers of liver and kidney damage, cell damage, clotting, blood glucose, and electrolytes were evaluated (See Figure 3 and Supplementary 1). Figure 3 summarizes the differences observed between NP and Low/High groups. White blood cells (WBC) count is increased in high ($10.9x10^3$, IQR 7.0–15.3) and low ($9.7x10^3$, IQR 6.8–14.6) IL-6 producers compared to NP ($8.2x10^3$, IQR 5.7–12.1) (Figure 3A). Interestingly, only high IL-6 producers presented significant changes in platelets count (40% of patients with high IL-6 levels are outside the reference values compared with 23% and 19% for NP and low producers, respectively). The median of platelets number is 168 (IQR 124-214) for high IL-6 producers, compared with 221 (IQR 171-271) and 219 (IQR 156-284) for low and NP, respectively (Figure 3B). On the other hand, the majority of low IL-6 producers are within the reference values for Lactate (95% compared to 73% for both NP and high IL-6 producers) (Figure 3C). Figure 3D summarizes the findings for C-reactive protein (CRP), where 85% of high and 78% of low IL-6 producers have altered values for CRP, a percentage above that found in non-producer patients (48%) for IL-6. In addition, the median of CRP levels is significantly higher in low (12.6 mg/L, IQR 5.6–68.5) and high (11.8 mg/L, IQR 6.1–15.3) IL-6 producers compared to NP (4.4 mg/L, IQR 1.5–9.8). We also evaluated the ability of lactate dehydrogenase (LDH) levels to identify different IL-6 producers. High IL-6 producers have increased levels of LDH (849.0 U/L, IQR 633.8 - 1107) compared to low (621.5 U/L, IQR 436.5 - 835.3) and NP (479.0 U/L, IQR 423.5 - 580.0). Thus, 92% of high IL-6 producers are outside the reference values, compared to 74% for low and 67% for NP (Figure 3E). Furthermore, the creatine kinase MB

isoenzyme (CKMB) is increased in high (27.0 U/L, IQR 11.0–90.0) and low (27.0 U/L, IQR 15.5–37.2) IL-6 producers compared to NP (16.6 U/L, IQR 11.5–22.0). Both high and low IL-6 producers have 53% of their patients outside CKMB reference values, a significant increase compared to NP group (13%) (Figure 3F).

Creatinine and urea, two renal markers, presented altered levels in low or high IL-6 producers. Figure 3G shows that Creatinine is increased in high IL-6 producers compared to NP. However, their median is within the reference range. In addition, there is no difference in numbers of patients with altered creatinine values between the groups. Regarding urea results, the median for low (48.9 mg/dL, IQR 34.7 – 81.0) and high (50.9 mg/dL, IQR 32.8 – 86.4) IL-6 producers are above the reference values, but only low IL-6 producers are statistically different from NP (41.1 mg/dL, IQR 31.4 – 69.7) (Figure 3H).

For analysis of possible predictors for IL-6 producer subtypes we used the Receiver Operating Characteristic Curve (ROC). The analyses of ROC curve shows that WBC values are able to differentiate low (> 8.63pg/mL; AUC = 0.60; sensitivity of 60.4% and specificity of 51.8%, LR 1.2; $p = 0.0024$) and high IL-6 producers (> 9.73 pg/mL; AUC = 0.62; sensitivity of 60.3% and specificity of 58.3%, LR 1.4; $p = 0.0060$) from NP (Figure 3A). Furthermore, platelet count is able to predict the group of high IL-6 producers compared to NP (< 193.5 pg/mL; AUC = 0.66; sensitivity of 60.0% and specificity of 59.0%, LR 1.4; $p = 0.0008$) (Figure 3B). Similar results are observed with the ROC curve, where Lactate values are able to only separate NP from low IL-6 producers (< 1.5 pg/mL; AUC = 0.70; sensitivity of 67.9% and specificity of 69.7%, LR 2.2; $p = 0.0007$) (Figure 3C). The CRP is a promise parameter to identify low (> 5.0 pg/mL; AUC = 0.74; sensitivity of 78.2% and specificity of 55.8%, LR 1.7; $p < 0.0001$) and high (> 5.8 pg/mL; AUC = 0.75; sensitivity of 82.3% and specificity of 61.0%, LR 2.1; $p < 0.0001$) IL-6 producers (Figure 3D). The LDH is also a significant biomarker for high IL-6 producers (> 577.5pg/mL; AUC = 0.83; sensitivity of 91.6% and specificity of 75.7%, LR 3.7; $p = 0.0006$) and a promise indicator of low IL-6 producers (> 491.5pg/mL; AUC = 0.63; sensitivity of 68.5% and specificity of 54.5%, LR 1.5; $p < 0.0325$) (Figure 3E). CKMB can also differentiate high (> 17.5 U/L; AUC = 0.70; sensitivity of 73.3% and specificity of 52.6%, LR 1.5; $p < 0.0226$) and low (> 16.6 U/L; AUC = 0.69; sensitivity of 72.5% and specificity of 50.0%, LR 1.4; $p < 0.0014$) IL-6 producers from NP (Figure 3F). Creatinine is only able to differentiate high IL-6 producers from NP (> 1.1U/L; AUC = 0.62; sensitivity of 62.7% and specificity of 63.0%, LR 1.7; $p < 0.0083$) (Figure 3G). Urea poorly distinguished high (> 43.3mg/dL; AUC = 0.59; sensitivity of 64.4% and specificity of 54.4%, LR 1.4; $p <$

0.0499) and low (> 42.4mg/d; AUC = 0.58; sensitivity of 60.1% and specificity of 5262; $p <$ 0.0116) IL-6 producers (Figure 3H).

Together, these results show that routine laboratory parameters are altered in high and low IL-6 producers, rendering them potential indicators of early IL-6 production (Figure 3I).

CRP is a potential biomarker to identify high IL-6 producers during COVID-19

Many laboratory parameters are altered in low and high IL-6 producers during COVID-19. However, principal component analysis (PCA) shows that these parameters are poorly able to define these populations (See supplementary figure 2). Then, using a multiple linear regression analysis, we sought to identify parameters most associated with IL-6 production. The results indicate that CRP ($p = 0.0052$) and LDH ($p = 0.0270$) are laboratory markers most associated with the production of IL-6 (Figure 4A). Based on the distribution of CRP (Gain ratio = 0.208) and LDH (Gain ratio = 0.070) values for each group, we observed that CRP is more efficient to identify NP, low and high IL-6 producers (Figure 4B, C). Next, we build a decision tree showing that CRP is a promise laboratory parameter able to define high IL-6 producers (Figure 4D). In addition, low platelet levels in high IL-6 producers may further contribute to identify this group (see supplementary figure 3). We also found a positive correlation between CRP levels with IL-6 production ($r = 0.3$, $p < 0.0001$) (Figure 4E). Finally, a multivariate logistic regression analysis with ROC curve revealed that the CRP/IL-6 axis is associated with non-survival of patients with COVID-19 (AUC = 0.78, $p < 0.0001$) (Figure 4F).

Taken together, the results presented herein show that subsets of patients with COVID-19 can be identified based on their levels of IL-6 production, which seems to play a role in the clinical manifestation of the disease. Furthermore, this study aimed to identify routine laboratory parameters as indicators of IL-6 levels, since they are frequently requested in public hospitals, as opposed to the quantification of IL-6. We show that routine laboratory parameters can be useful in determine high IL-6 producers early in the development of COVID-19, especially CRP and platelets. This can be crucial to manage the choice of therapy and improve patients prognosis.

Discussion

The cytokine IL-6 is a key mediator in the outcome of COVID-19, despite great advances in the knowledge of the disease, little is known about the different producers of IL-6 (Han et al., 2020; Tay et al., 2020). Knowing the need and difficulty of detect these producers

in public hospitals, in this work we characterized and show possible laboratory markers capable of helping in the identification of subpopulations of IL-6 producers in COVID-19.

Shortly after the World Health Organization (WHO) declared COVID-19 a pandemic disease, the cytokine IL-6 was already described as a possible good biomarker for the most severe form of the disease (Wang et al., 2020). The development of severe COVID-19 is characterized by the need for intensive care, mechanical ventilation and greater lung damage, these worse outcomes have been associated with the production of IL-6 in this and other studies (Broman et al., 2021; Coomes & Haghbayan, 2020; Gubernatorova et al., 2020; Han et al., 2020; Moore & June, 2020). We found that with advancing age, patients with COVID-19 appear to produce higher levels of IL-6. It is known that healthy individuals tend to increase by 0.05 pg/mL every 1 year of age. Indeed, IL-6 production and advanced age are risk factors for severe COVID-19 (Said et al., 2021; F. Zhou et al., 2020).

In a clinical study, levels above 30 pg/mL of IL-6 were sufficient to consider the need for mechanical ventilation in patients with COVID-19 (Galván-Román et al., 2021). In search of values that can be used as biomarkers in clinical practice, Liu and colleagues showed that IL-6 production above 32.1 pg/mL was related to severe COVID-19 (Liu et al., 2020a). On the other hand, a meta-analysis showed that the range of IL-6 production in healthy subjects was from 0 to 43.5 pg/mL (Said et al., 2021). This reinforces the need to characterize subgroups of IL-6 producers to understand the range of production in a specific disease.

With strong evidence that IL-6 is one of the main inflammatory mediators for the worst prognosis of COVID-19, it did not take long for the medical and scientific community to test drugs capable of inhibiting the effect of this mediator. For a beneficial outcome for the patient with COVID-19, the prescription of therapies to block the action of IL-6 must be carefully analyzed, observing the patient's health status, the severity of the disease and the intervention time, since, results are shown to be heterogeneous (Honore et al., 2020; Jones & Hunter, 2021; Matthay & Luetkemeyer, 2021).

Although we are scientifically convinced of the importance of IL-6 levels in the outcome of COVID-19, the measurement of IL-6 production is not routine in public hospitals in underdeveloped or developing countries, so cheaper and more sensitive alternatives to identify these producers are welcome in current and future pandemics by coronavirus. This is the first study that seeks to identify subpopulations of IL-6 producers using routine laboratory markers. We found different laboratory markers capable of distinguishing non, low and high producers. Changes in blood laboratory markers are used as a predictor for severe COVID-19, however,

the objective of this study is to identify which ones are most associated with the production of IL-6, a key mediator for the worst prognosis and possible therapeutic target (Gallo Marin et al., 2021; Guan et al., 2020; Velavan & Meyer, 2020; Wu et al., 2021; Zhang et al., 2020). In this context, systemic levels of CRP proved to be the best biomarker for IL-6 production. The induction of CRP by IL-6 is already known in the field of immunology, we showed that the CRP/IL-6 axis is related to non-survival of patients with COVID-19 (del Giudice & Gangestad, 2018; Liu et al., 2020b; Y. Z. Zhou et al., 2021). Although less associated with IL-6 production than CRP, LDH is also associated with mediator production and is present in severe cases of COVID-19 (Huang et al., 2020; Zhang et al., 2020).

Together, our findings show that there are different subgroups of IL-6 producers in COVID-19, which have different clinical characteristics and outcomes. We have shown that there is a relationship between increased IL-6 levels and worse COVID-19 outcomes. Furthermore, we identified that laboratory markers such as WBC, platelets, lactate, LDH, CKMB and especially CRP can be used to help identify subgroups of IL-6 producers. In addition to strategically helping to prescribe IL-6 inhibitors indirectly, the findings of this study facilitate the identification of possible producers of IL-6 in a simple, routine and inexpensive way, especially for hospitals with low resources.

Authorship

I.B.S., T.C.S., S.N., R.K., P.R.S.O., A.B., R.T., C.B., M.B-N., V.B., and N.M.T. contributed to the article's writing or substantial involvement in its revision before submission. M.R.S.C., and J.R.C. conducted the medical care of the research participants. I.B.S., J.S., S.N., A.I. and L.E. processed the biological samples and performed the laboratory essays. I.B.S., contributed to the acquisition of the data or the analysis and interpretation of information. I.B.S., N.M.T., and V.B.B. were involved in the study's conception, hypotheses delineation, and design. N.M.T. is this work's guarantor. She had full access to all the study's data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

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Tables

Table 1. Clinical characteristics of individuals with Coronavirus Disease 2019 (COVID-19) no, low or high IL-6 producers

	Patients			
CHARACTERISTICS	Non Producers (n = 127)	Low (n = 215)	High (n = 63)	*p value*
Male, n (%) N	70 (55,1%) 127	113 (52,5%) 215	30 (47,6%) 63	0.6218
Age, median (IQR) ** N	59 (40-74) 112	65 (54-77) 198	68 (59-81) 55	0.0016
Symptoms onset to admission (days), median (IQR) N	6 (4-10) 92	8 (5-11) 162	7 (5-11) 46	0.0811
IL-6 levels (pg/mL), median (IQR) ****	0 (0-0)	26,3 (15,7-51,3)	319,8 (174,6-490,1)	<0.0001
Comorbidities n/N (%)				
Obesity	22/86 (25,5%)	42/166 (25,3%)	11/41 (26,8%)	0.9801
Diabetes	42/95 (44,2%)	82/166 (49,3%)	16/40 (40,0%)	0.4867
Hypertension	65/102 (60,8%)	107/170 (63,0%)	33/47 (70,2%)	0.6448
COPD	9/77 (11,7%)	11/134 (8,2%)	5/38 (13,1%)	0.5660
Smoker	23/85 (27,0%)	48/143 (33,5%)	12/38 (31,6%)	0.5900
Heart disease *	22/78 (28,2%)	17/133 (12,8%)	5/38 (13,1%)	0.0131
Kidney disease	14/86 (16,2%)	18/141 (12,7%)	5/41 (12,1%)	0.7189
Cancer	6/76 (7,9%)	7/134 (5,2%)	2/37 (5,4%)	0.7261

Symptoms n/N (%)				
Fever	47/99 (47,5%)	101/165 (61,2%)	24/43 (55,9%)	0.0934
Cough	73/105 (69,5%)	125/171 (73,1%)	31/44 (70,4%)	0.8029
Dyspnoea	69/108 (63,9%)	117/176 (66,5%)	31/49 (63,3%)	0.8654
Headache *	23/85 (27,0%)	21/133 (15,8%)	4/39 (10,2%)	0.0391
Myalgia **	38/87 (43,7%)	43/138 (31,1%)	6/37 (16,2%)	0.0091
Fatigue	27/86 (31,4%)	41/142 (28,9%)	9/40 (22,5%)	0.5891
Diarrhea	16/83 (19,3%)	24/137 (17,5%)	5/38 (13,1%)	0.7121
Taste or smell dysfunction	12/82 (14,6%)	23/137 (16,8%)	7/38 (18,4%)	0.8542

COPD = Chronic Obstructive Pulmonary Disease; n = Sample size; N = Number available; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$

Figures

Figure 1

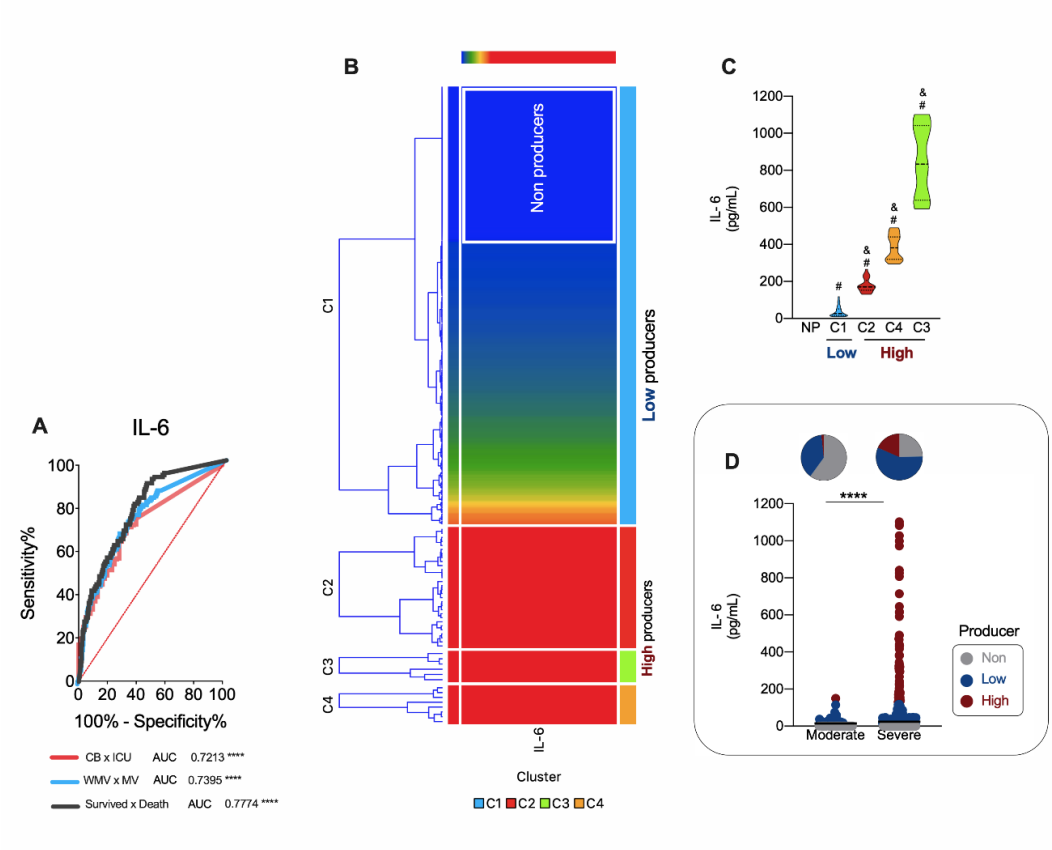


Figure 2

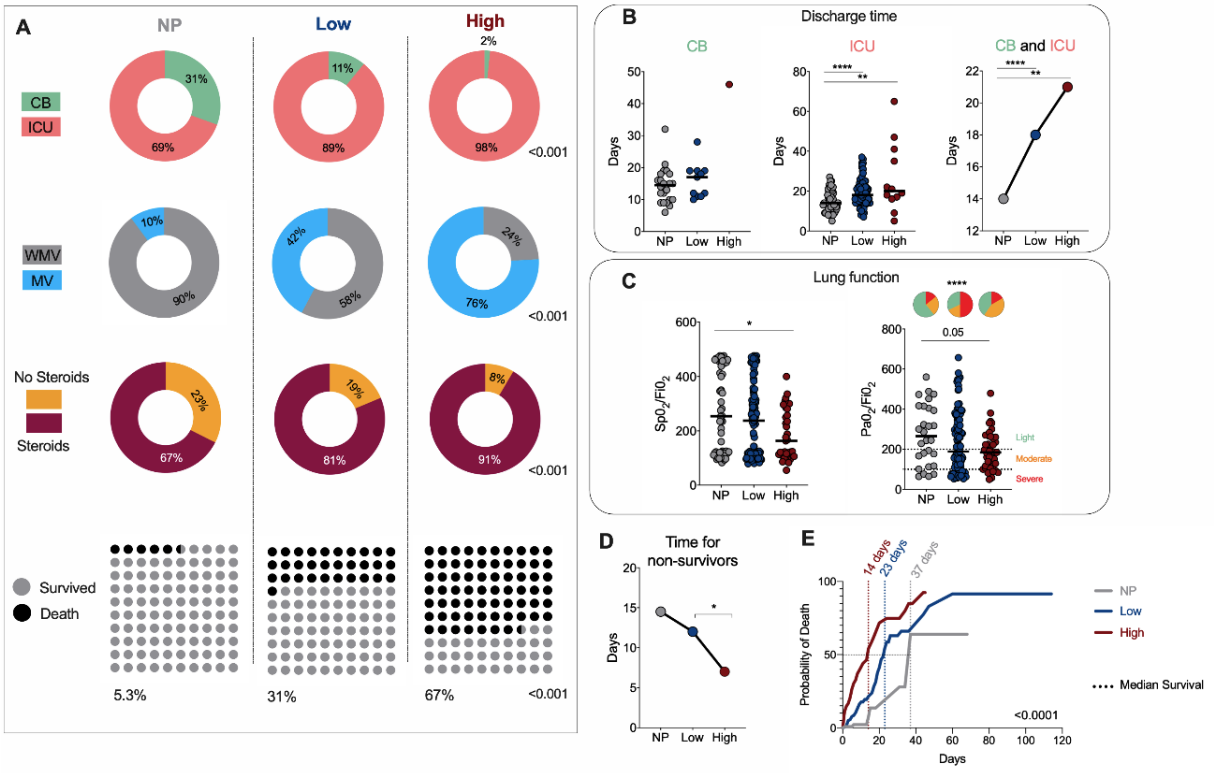

A
NP
Low
High
CB
ICU
31%
69%
11%
89%
2%
98%
<0.001
WMV
MV
10%
90%
42%
58%
24%
76%
<0.001
No Steroids
Steroids
23%
67%
19%
81%
8%
91%
<0.001
Survived
Death
5.3%
31%
67%
<0.001
B
Discharge time
CB
ICU
CB and ICU
Days
NP Low High
C
Lung function
SpO2/FiO2
PaO2/FiO2
0.05
Light
Moderate
Severe
D
Time for non-survivors
E
14 days
23 days
37 days
Probability of Death
<0.0001
Days
NP
Low
High
Median Survival

Figure 3

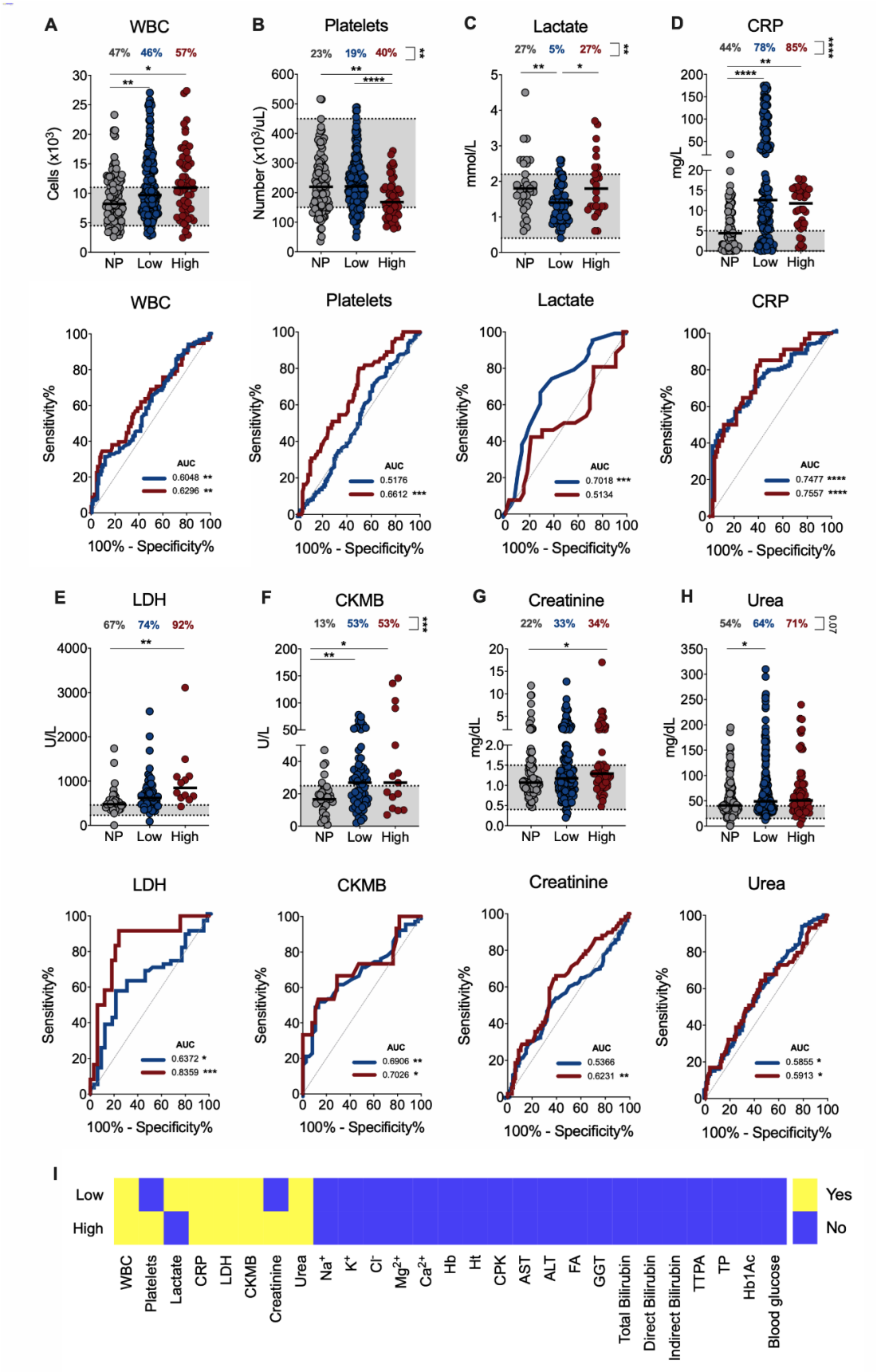
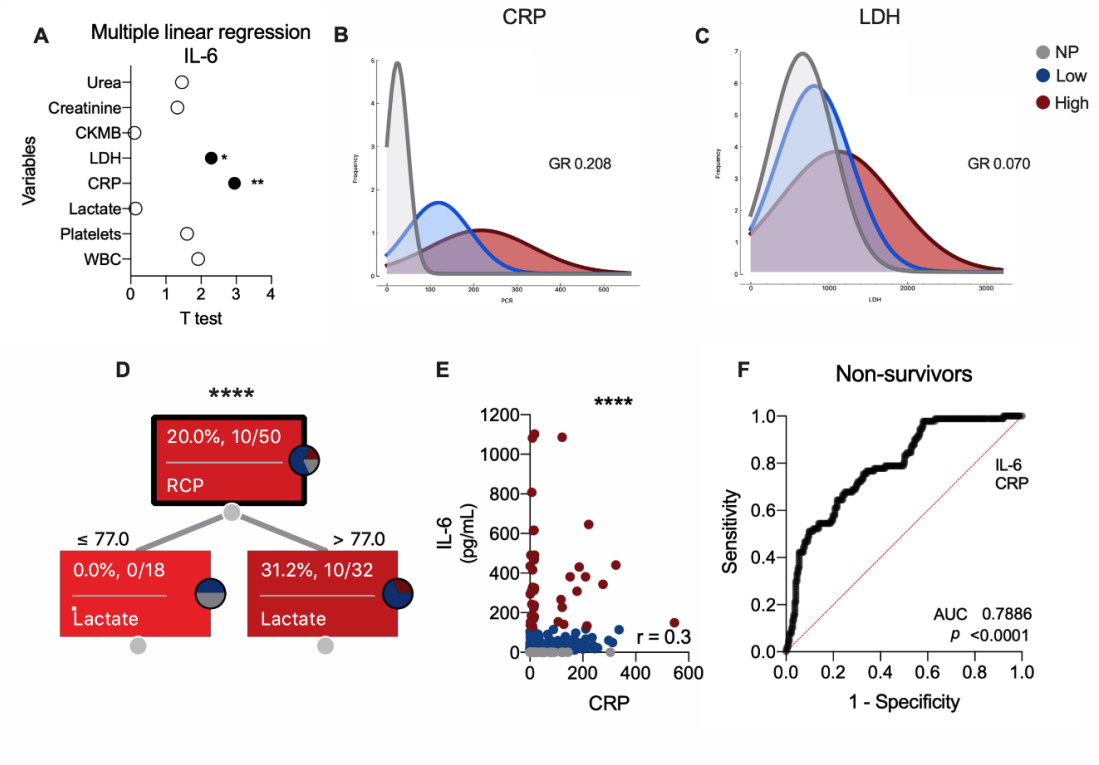
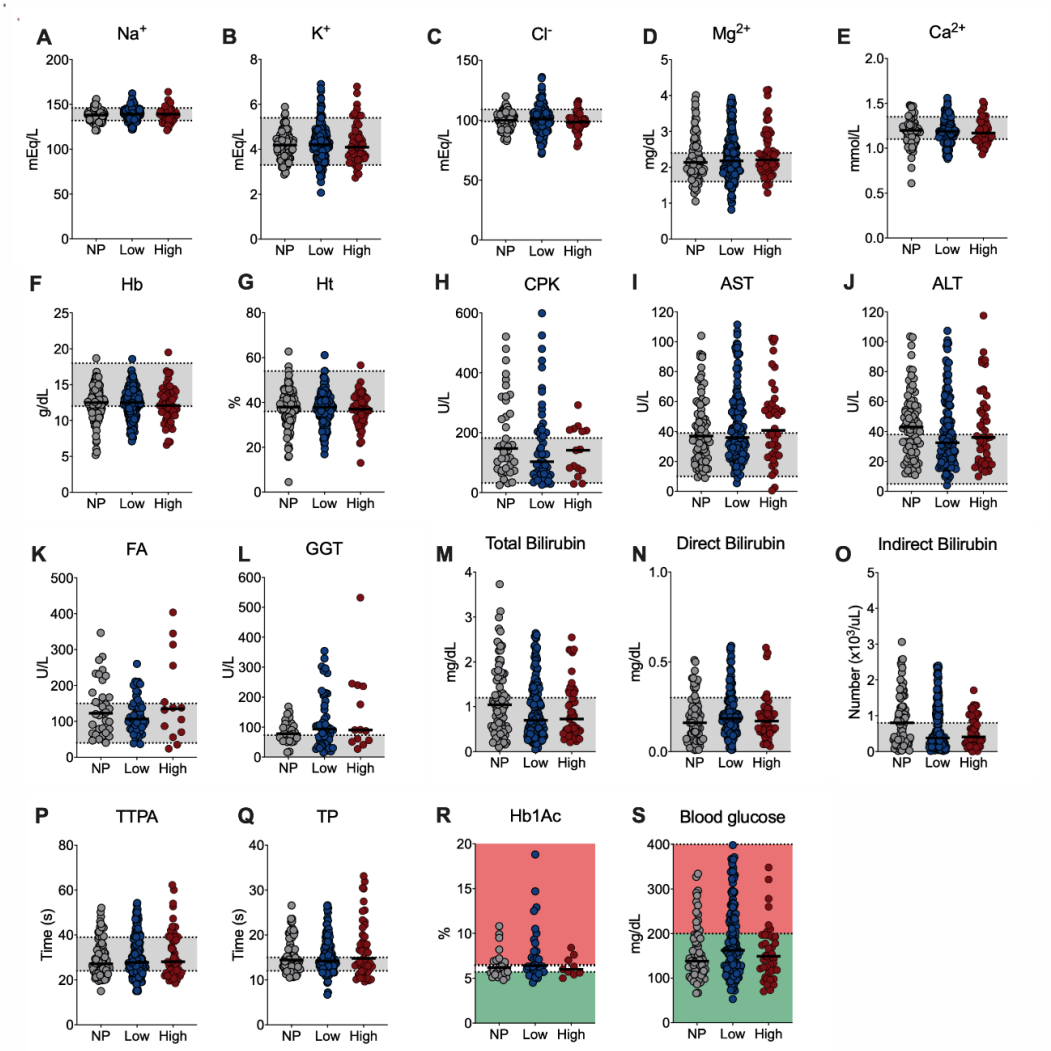

A
WBC
47% 46% 57%
Cells (x10³)
NP Low High
B
Platelets
23% 19% 40%
Number (x10³/uL)
C
Lactate
27% 5% 27%
mmol/L
D
CRP
44% 78% 85%
mg/L
WBC
Platelets
Lactate
CRP
Sensitivity%
100% - Specificity%
AUC
0.6048 **
0.6296 **
0.5176
0.6612 ***
0.7018 ***
0.5134
0.7477 ****
0.7557 ****
E
LDH
67% 74% 92%
U/L
F
CKMB
13% 53% 53%
G
Creatinine
22% 33% 34%
mg/dL
H
Urea
54% 64% 71%
0.07
LDH
0.6372 *
0.8359 ***
CKMB
0.6906 **
0.7026 *
Creatinine
0.5366
0.6231 **
Urea
0.5855 *
0.5913 *
I
Low
High
Yes
No
WBC
Platelets
Lactate
CRP
LDH
CKMB
Creatinine
Urea
Na+
K+
Cl-
Mg2+
Ca2+
Hb
Ht
CPK
AST
ALT
FA
GGT
Total Bilirubin
Direct Bilirubin
Indirect Bilirubin
TTPA
TP
Hb1Ac
Blood glucose

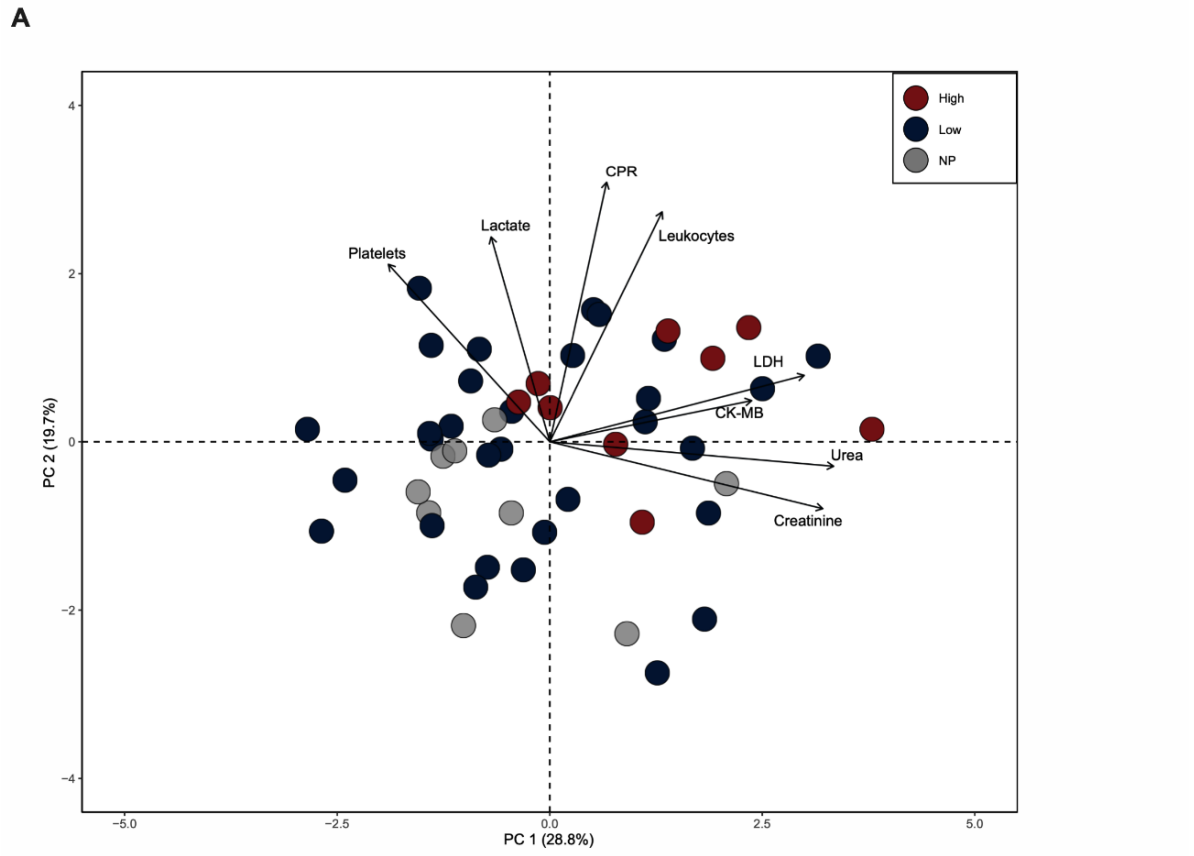
Figure 4


A
Multiple linear regression
IL-6
Urea
Creatinine
CKMB
LDH
CRP
Lactate
Platelets
WBC
Variables
*
**
0 1 2 3 4
T test
B
CRP
GR 0.208
C
LDH
GR 0.070
NP
Low
High
D
****
20.0%, 10/50
RCP
≤ 77.0
> 77.0
0.0%, 0/18
Lactate
31.2%, 10/32
Lactate
E
****
IL-6 (pg/mL)
1200
1000
800
600
400
200
0
r = 0.3
0 200 400 600
CRP
F
Non-survivors
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
IL-6
CRP
AUC 0.7886
p <0.0001
0.0 0.2 0.4 0.6 0.8 1.0
1 - Specificity

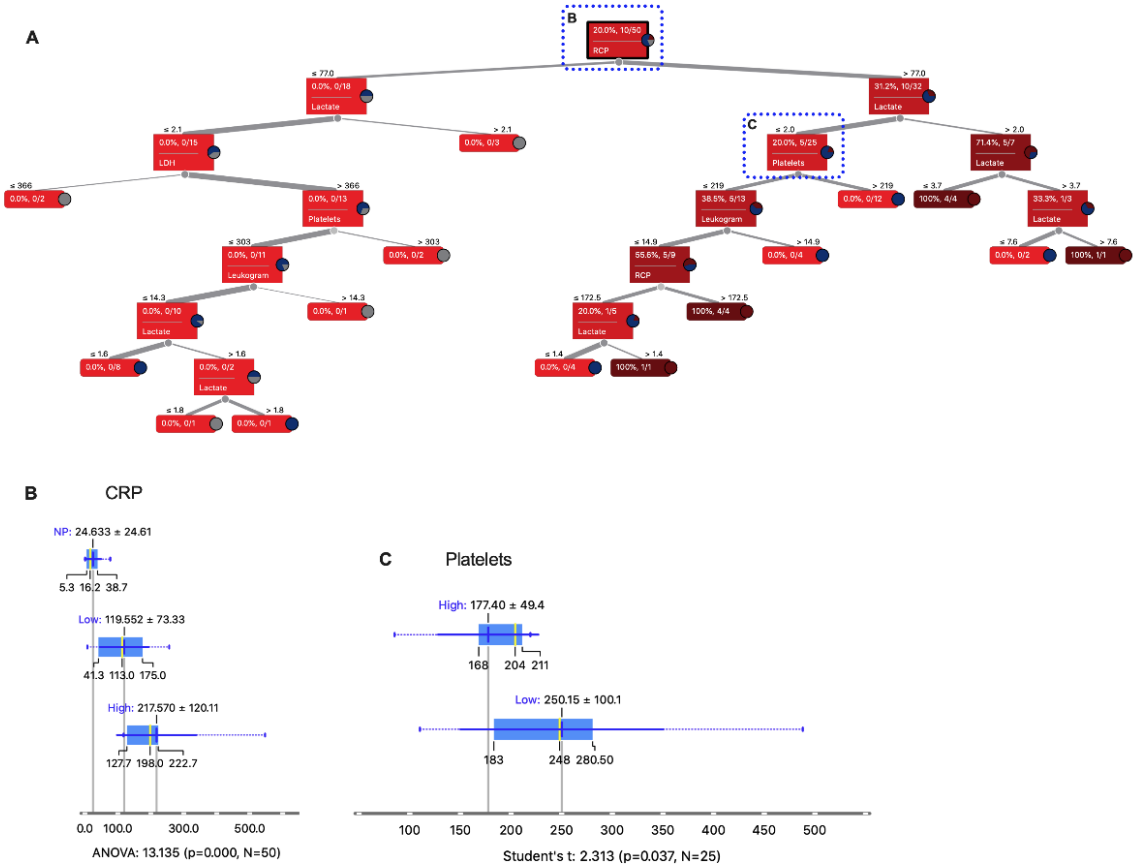
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Figures legends

Figure 1. No, Low or High producers of systemic IL-6 levels are found in COVID-19. (A) Receptor Operation Characteristic Curve (ROC curve) of IL-6 levels between clinical beds (CB) and intensive care unit (ICU) (red), need for mechanical ventilation (MV) or not (WMV) (blue) and survival or death (gray) in COVID-19 patients. (B) Heatmap unsupervised showing different clusters based on IL-6 production. (C) Violin Plot truncated showing median and quartiles of IL-6 production in the clusters found. (D) Scatter plot showing the presence of No, Low or High producers based on disease severity. Data shown in median. C, Kruskal-Wallis test. D, Mann-Whitney test. # difference statistic compared with NP; & compared with C1. ****$p < 0.0001$.

Figure 2. High IL-6 producers develop more severe COVID-19. (A) Panel with percentage of patients from the NP, Low and High group who required ICU, mechanical ventilation, steroids and who did not survive. (B) Time from symptom onset to hospital discharge in CB, ICU or both in NP, Lor and High group. (C) SpO2/fiO2 and PaO2/FiO2 ratio of admission in NP, Low and High group. (D) Median length of stay until death in NP, Low and High group. (E) Probability of death due to COVID-19 in NP, Low or High patients. Data shown in median. A, C, Chi-square test. B-D, Kruskal-Wallis. E, Log-rank test. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

Figure 3. Elevated production of IL-6 induced by COVID-19 is accompanied by changes in laboratory parameters. Quantification (above) and ROC curve generation (below) of (A) White blood cells, (B) Platelets, (C) Lactate, (D) C-reactive protein, (E) Lactate dehydrogenase, (F) MB isoform creatine kinase, (G) Creatinine, (H) Urea from blood samples of NP, Low and High patients. (I) Heatmap showing the laboratory parameters capable of identifying (yellow) low or high producers in relation to non-producers. Data shown in median. Kruskal-Wallis. Chi-square test. AUC = Area Under the Curve. Hb = Hemoglobin. Ht = hematocrit. TTPA = Activated Partial Thromboplastin Time. TP = Prothrombin Time. Hb1Ac = Glycated hemoglobin. AST = Aspartate aminotransferase. ALT = Alanine aminotransferase. Gray range = reference values. The percentages refer to the number of patients outside the reference values. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure 4. CRP is the best biomarker for high IL-6 producers in COVID-19. (A) Multivariate linear regression analysis of laboratory parameters and IL-6 production in all COVID-19 patients. Frequency distribution of (B) CPR and (C) LDH production in No, Low and High IL-6 producers. Correlation between IL-6 production with (D) CRP and (E) LDH. (F) Multiple logistic regression between IL-6, CRP and LDH with death in COVID-19 patients. (G) Decision tree with laboratory parameters targeting high IL-6 producers. *r* = Spearman r correlation. G, ANOVA. Gray = Non-producers; Blue = Low producers; Red = High producers. GR = Gain ration. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure S1. Laboratory parameters in No, Low or High producers. Values of (A) Sodium, (B) Potassium, (C) Chlorine, (D) Magnesium, (E) Calcium, (F) Hemoglobin, (G) Hematocrit, (H) Creatine Phosphokinase, (I) Aspartate Aminotransferase, (J) Alanine Aminotransferase, (K) Alkaline phosphatase, (L) Gamma-glutamyl Transferase, (M) Total Bilirubin, (N) Direct Bilirubin, (O) Indirect Bilirubin, (P) Activated Partial Thromboplastin Time Assay, (Q) Prothrombin time, (R) Glycated hemoglobin and (S) Blood glucose. Gray region = limit of reference values. Green area = no diabetes; white = prediabetes; red = diabetes.

Figure S2. Principal component analysis with different laboratory parameters. (A) Principal component analysis (PCA) with laboratory parameters of No, Low and High producers. Gray = Non-producers; Blue = Low producers; Red = High producers.

Figure S3. Complete decision tree for high IL-6 producers. (A) Decision tree for high IL-6 producers. Comparison test between No, Low and High producers in relation to the (B) CRP and (C) Platelets node. Blue dotted box = nodes with statistical difference.

6 **DISCUSSÃO**

O SARS-CoV-2 se espalhou rapidamente por todo o mundo, com um elevado número de pessoas infectadas e óbitos, e, por isso, a pandemia da COVID-19 é considerada uma das mais devastadoras enfermidades provocadas por coronavírus. Devido ao grande número de infectados, a identificação de mecanismos e fatores de risco envolvidos no agravamento da COVID-19 se tornou de extrema importância para minimizar o impacto da doença emergente. Sabe-se que o diabetes é um dos principais fatores de risco para a COVID-19 grave. No entanto, ainda são pouco conhecidos os mecanismos envolvidos nesse agravamento (MUNIYAPPA; GUBBI, 2020; PINTO et al., 2020; SHI et al., 2020). No presente estudo, nós buscamos identificar os mediadores induzidos naturalmente pelo diabetes e pré-diabetes, que tenham capacidade de piorar o quadro clínico de pacientes com COVID-19.

Nossos resultados mostram que o diabetes é induz a expressão de genes envolvidos na síntese e reconhecimento do LTB_4 em PBMCs. Além disso, durante a COVID-19, pacientes com diabetes apresentam um aumento na expressão de receptores envolvidos na internalização do SARS-CoV-2 (ECA2/TMPRSS2), assim como, aumento da produção de LTB_4. Também foi demonstrado que o aumento da expressão de ALOX5, enzima responsável pela síntese de LTB_4, e ECA2 estava relacionado com a gravidade da COVID-19.

Apesar de pouco compreendido o papel do LTB_4 na COVID-19, esse mediador lipídico participa de diversas desordens respiratórias, como inflamação, pneumonia, síndrome do desconforto respiratório e lesão pulmonar aguda (BIERNACKI; KHARITONOV; BARNES, 2003; BRANDT; SEREZANI, 2017; EUN et al., 2012; MASCLANS et al., 2007; MUNIYAPPA; GUBBI, 2020; TAY et al., 2020). O envolvimento do LTB_4 no diabetes tem sido estudado em modelo experimental. Sabe-se que o diabetes espontaneamente induz a produção de LTB_4, o que tem sido associado a suscetibilidade a infecções, dificuldade de cicatrização e aumento da resistência a insulina (BRANDT et al., 2018; FILGUEIRAS et al., 2015a; LI et al., 2015).

Durante a infecção pelo SARS-CoV-2, algumas moléculas são necessárias durante a interação vírus-hospedeiro, dentre elas ECA2 e TMPRSS2 no hospedeiro, devido a sua afinidade com a proteína Spike do vírus (RADZIKOWSKA et al., 2020; TAY et al., 2020). Em condições grave da COVID-19, monócitos são constantemente recrutados para o sítio de infecção e alta concentração de glicose aumenta a expressão de ECA2 em monócitos humanos, bem como a carga viral do SARS-CoV-2 (CODO et al., 2020). O pulmão, principal órgão

acometido pela COVID-19, apresenta aumento da expressão de ECA2 em indivíduos com diabetes (PINTO et al., 2020; WIJNANT et al., 2020). Ao analisar indivíduos diabéticos com COVID-19, nosso grupo demonstrou que um aumento da expressão de ECA2 e TMPRSS2 em PBMCs, além de uma tendência de aumento no pulmão, o que podem configurar maior suscetibilidade ao SARS-CoV-2 (CODO et al., 2020; WIJNANT et al., 2020).

Nossos achados mostram que PBMCs de indivíduos com diabetes possuem uma correlação positiva entre ECA2 e ALOX5. Esta relação ainda é pouco compreendida na literatura, apesar de isso não demonstrar um efeito de casualidade, essas vias reguladas positivamente de forma independente, podem contribuir para o aumento da suscetibilidade e potencialização da inflamação no contexto da COVID-19 (BRANDT; SEREZANI, 2017; CODO et al., 2020).

A infecção e subsequente inflamação desencadeada pelo SARS-CoV-2 provoca diversos sintomas. Nesse cenário, a febre, tosse e falta de ar, são os sintomas mais relatados por indivíduos que foram acometidos pela COVID-19. Assim como em um estudo feito em Wuhan, nós identificamos que pacientes com diabetes e COVID-19 possuem maior frequência de falta de ar (SHI et al., 2020). Bem como o diabetes, a hipertensão também é fator de risco associado a COVID-19 grave. Os pacientes da nossa coorte, assim como de outros estudos, frequentemente apresentam diabetes e hipertensão, devido à características intrínsecas da população (MUDATSIR et al., 2020; SARDU et al., 2020; SHI et al., 2020; WIJNANT et al., 2020). Apesar de não encontrarmos aumento de mortalidade por complicações da COVID-19 em pacientes com diabetes, assim como relatado em outros estudos, nós encontramos que a doença é mais grave nesses indivíduos, com maior duração e desconforto respiratório (CHEN et al., 2020; MUNIYAPPA; GUBBI, 2020; WANG et al., 2020a).

A gravidade da COVID-19 tem sido associada com a tempestade de citocinas ativada pelo SARS-CoV-2 (TAY et al., 2020). Além da IL-6, uma das mais relacionadas com a COVID-19 grave, os mediadores inflamatórios IL-1b, IFN-γ, MCP-1, CCL2, CXCL10 e TNF-a também possuem forte influência no desfecho da doença (HAN et al., 2020; MUNIYAPPA; GUBBI, 2020; TAY et al., 2020). Apesar do escasso conhecimento sobre mediadores lipídicos na COVID-19, nosso trabalho mostrou que o LTB_4 induzido pelo diabetes também pode contribuir para o agravamento da doença, uma vez que ele é um potente indutor de inflamação (BRANDT; SEREZANI, 2017; SEREZANI et al., 2011). Corroborando com nossos achados, um estudo mostrou a produção de mediadores lipídicos na COVID-19 associada a gravidade da doença, bem como, o resultado da utilização de terapias com glicocorticoides nesses pacientes

(PÉREZ et al., 2021). Em outras doenças pulmonares não associadas a coronavírus, o LTB_4 tem sido mais estudado. Em modelo experimental de lesão pulmonar aguda, o LTB_4 foi detectado em altos níveis no lavado broncoalveolar (EUN et al., 2012). Em indivíduos com Doença Pulmonar Obstrutiva Crônica (DPOC), a gravidade da doença foi associada com altos níveis de LTB_4 exalados no ar (BIERNACKI; KHARITONOV; BARNES, 2003). O LTB_4 também se mostrou relevante na Síndrome do Desconforto Respiratório Agudo (SDRA) (MASCLANS et al., 2007). Nesse sentido, indivíduos com SDRA exibiram até 5 vezes mais níveis de LTB_4 comparado a indivíduos controle (DAVIS et al., 1989). Isso mostra que o LTB_4, apesar de ainda pouco explorado na COVID-19, já foi associado a complicações respiratórias na literatura.

O papel do LTB_4 na inflamação, além de induzir outros mediadores inflamatórios, é a grande capacidade quimiotática para neutrófilos, condição presente na COVID-19 (BRANDT et al., 2018; ZHANG et al., 2020). Um estudo produzido por nosso grupo mostrou que a produção de LTB_4 também tem sido relacionada com a ativação de inflamassomas. Estes últimos parecem ter participação na piora do prognóstico da COVID-19 (RODRIGUES et al., 2020; SALINA et al., 2020). Diferentemente do encontrado com a expressão do gene ALOX5 no nosso estudo, os níveis séricos de LTB_4 não apresentaram associação com o prognóstico da COVID-19. Isso pode ser devido ao tipo de amostra analisada, uma vez que a expressão gênica foi proveniente de monócitos e linfócitos (PBMCs), enquanto a amostra de plasma contém a produção de LTB_4 também de outros tipos celulares, como neutrófilos, uma das principais células produtoras desse mediador lipídico (BRANDT; SEREZANI, 2017). No entanto, o LTB_4 sistêmico pode contribuir de diferentes maneiras para a inflamação, como aumento da quimiotaxia e potencialização inflamatória (AFONSO et al., 2012; BRANDT; SEREZANI, 2017).

Diante de uma doença onde a inflamação exacerbada e pouco limitada é um problema, atualmente, as principais alternativas terapêuticas para a COVID-19 envolvem a supressão da ação de mediadores lipídicos direta ou indiretamente. O estudo RECOVERY (Randomised Evaluation of COVID-19 Therapy) mostrou que a utilização de dexametasona diminuiu a taxa de mortalidade de pacientes com COVID-19 grave (BUILDING; CAMPUS; DRIVE, 2020). Além disso, a utilização de montelucaste e outros inibidores relacionados a inibição da via do ácido araquidônico são propostos para amenizar as complicações da COVID-19 (PÉREZ et al., 2021; SANGHAI; TRANMER, 2020).

Ao analisar os mediadores inflamatórios e desfecho da doença, um grupo de pacientes chamou a atenção de nosso grupo, se tratava de pacientes com valores alterados de glicemia, no entanto, dentro do limite para o diagnóstico do diabetes. Sabe-se que a cada dez adultos, um desenvolverá diabetes nos próximos anos, mas muitos desses indivíduos já possuem o risco aumentado, denominado pré-diabetes (IDF, 2015). Já é amplamente conhecido e consolidado que o diabetes é fator de risco para a COVID-19 grave. No entanto, os dados de indivíduos com pré-diabetes acometidos pela doença ainda são escassos na literatura. Estudos recentes tem demonstrado que 6% a 39,4% de pacientes com pré-diabetes desenvolvem a COVID-19 grave (SATHISH; CHANDRASEKARAN, 2021; SMITH et al., 2021; VARGAS-VÁZQUEZ et al., 2021). Dentre esse campo pouco explorado, nossos dados sugerem que o pré-diabetes também pode induzir o agravamento da COVID-19 principalmente devido a alta produção da IL-6.

A citocina IL-6 é um dos mediadores chave na tempestade de citocinas decorrente da COVID-19, estando constantemente relacionado com a gravidade da doença. A idade, o tipo de internamento e a necessidade de ventilação mecânica são alguns dos fatores que estão associados com a alta produção desse mediador. O atual estudo também buscou avaliar características clínicas de subpopulações de IL-6, bem como, possíveis marcadores laboratoriais os quais possam auxiliam na identificação de forma precoce (SAID et al., 2021; ZHOU et al., 2020a). Independente do risco aumentado para o diabetes ou o diabetes diagnosticado, a proteína C reativa (PCR), relatada por nosso estudo, parece ser o melhor marcador laboratorial para a identificação de altos produtores de IL-6 na COVID-19, relação esta, que já é bem compreendida na área da imunopatologia (DEL GIUDICE; GANGESTAD, 2018).

Apesar das limitações amostrais, nosso estudo reportou o aumento da produção de IL-6 sérica induzida pelo pré-diabetes na COVID-19, o que tem sido amplamente discutido em outros contextos, independente do nível glicêmico (BONYEK-SILVA et al., 2021; HAN et al., 2020; TAY et al., 2020). Diferentemente do que encontramos em pacientes com diabetes e COVID-19, os níveis de LTB_4 não parecem influenciar a gravidade da doença em indivíduos com pré-diabetes (BONYEK-SILVA et al., 2021). Mas, assim como em indivíduos com diabetes, as razões de PaO_2/FiO_2 e SpO_2/FiO_2 induzidas pela COVID-19 em indivíduos com pré-diabetes são menores em comparação a indivíduos sem diabetes (BONYEK-SILVA et al., 2021). Em conjunto, nossos dados evidenciam uma maior lesão pulmonar induzida não só pelo diabetes, mas também em indivíduos com pré-diabetes acometidos pela COVID-19.

Após a fase aguda da doença, alguns pacientes apresentam sintomas residuais persistentes, denominado de COVID-19 longa. Estes sintomas podem durar por meses e tem preocupado as equipes médicas em todo mundo (HUANG et al., 2021; LOPEZ-LEON et al., 2021). Dados similares ao nosso estudo também reportaram que o diabetes não induz um perfil de sintomas na fase pós-COVID-19 diferente daqueles observados em não-diabéticos (FERNÁNDEZ-DE-LAS-PEÑAS et al., 2021). Nossos achados demonstram que os sintomas mais relatados na COVID-19 longa são dispneia, fadiga, cefaleia, tosse e dor no corpo, registros que também foram relatados em outros estudos (DENNIS et al., 2021; HUANG et al., 2021; LOPEZ-LEON et al., 2021). Assim, apesar de possuírem uma fase aguda mais grave, pacientes com pré-diabetes ou diabetes parecem ter sequelas decorrentes da COVID-19 similares a indivíduos sem diabetes.

7 **CONCLUSÃO**

Os resultados desse estudo demonstram que alterações observadas em pacientes pré-diabéticos ou diabéticos com COVID-19 podem favorecer a evolução para a forma grave da doença. No entanto, apesar de ambos os grupos evoluírem para a forma grave da COVID-19, os mecanismos envolvidos não parecem ser compartilhados. No diabetes, a ativação da via do LTB_4, níveis elevados de IL-6 e o aumento da expressão de genes relacionados a invasão da célula hospedeira pelo SARS-CoV-2 estão relacionados com a gravidade da COVID-19. Por outro lado, dentre os mediadores analisados, apenas a produção de IL-6 observada em indivíduos com pré-diabetes tem papel no agravamento da COVID-19 durante a fase aguda, mas não a longo prazo. Esse estudo também mostrou que independentemente do nível glicêmico, altos produtores de IL-6 estão relacionados com o pior desfecho da COVID-19 e podem ser identificados por meio de exames laboratoriais de rotina. Portanto, independente do mecanismo, nossos estudos indicam que a inflamação crônica encontrada em indivíduos com pré-diabetes ou diabetes pode influenciar o desfecho da COVID-19.

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Anexo A - Parecer de aprovação do CEP

HOSPITAL SANTO ANTÔNIO/
OBRAS SOCIAIS IRMÃ DULCE

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Resposta imune e inflamatória pós hospitalização dos pacientes acometidos com COVID-19: Estudo prospectivo observacional.
Pesquisador: JULIANA CALDAS RIBEIRO BITTENCOURT
Área Temática:
Versão: 3
CAAE: 33366020.5.0000.0047
Instituição Proponente: BAHIA SECRETARIA DE SAUDE DO ESTADO
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.233.237

Apresentação do Projeto:
INTRODUÇÃO
No início de dezembro de 2019, em Wuhan, capital da província de Hubei, foram identificados os primeiros casos de uma pneumonia de etiologia desconhecida. (1) A maioria dos pacientes trabalhavam ou moravam no mercado atacadista local de frutos do mar de Huanan, onde também estavam à venda animais vivos. (5) Nos estágios iniciais desta pneumonia, sintomas de infecção respiratória aguda grave foram encontradas, com alguns pacientes desenvolvendo rapidamente síndrome do desconforto respiratório agudo (SDRA),insuficiência respiratória aguda e outras complicações graves. (5) Em 7 de janeiro, um novo coronavírus foi identificado pelo Centro Chinês para Controle e Prevenção de Doenças (CDC) a partir da amostra de esfregaço da cavidade oral de um paciente e posteriormente foi nomeado 2019-nCoV pela OMS. (6) O patógeno foi identificado como um novo RNA de betacoronavírus, (7) atualmente denominado coronavírus 2 da síndrome respiratória aguda grave (SARSCoV-2), que possui uma similaridade filogenética ao SARSCoV2. (8) A Organização Mundial da Saúde (OMS) declarou recentemente a doença pelo coronavírus 2019 (Covid-19) uma emergência de saúde pública de interesse internacional. (9) Final de fevereiro, um total de
81.109 casos confirmados em laboratório havia sido documentado globalmente. (10), (11), (9), (12). A taxa de letalidade por esse vírus, estimada pela Organização Mundial de Saúde (OMS), é de 3,4% (13). Essa taxa de letalidade é semelhante à da influenza espanhola (2 a 3%) (14) e muito

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Telefone: (71)3310-1335 **Fax:** (71)3310-1335 **E-mail:** cep@irmadulce.org.br

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: A resposta imune humoral na infecção pelo SARS-CoV-2 e sua potencial aplicação clínica.

Pesquisador: Natalia Machado Tavares

Área Temática:

Versão: 2

CAAE: 36199820.6.0000.0040

Instituição Proponente: Centro de Pesquisas Gonçalo Moniz - CPqGM/ FIOCRUZ/ BA

Patrocinador Principal: Centro de Pesquisas Gonçalo Moniz - CPqGM/ FIOCRUZ/ BA
FIOTEC - FUNDAÇÃO PARA O DESENVOLVIMENTO CIENTIFICO E TECNOLOGICO EM SAUDE

DADOS DO PARECER

Número do Parecer: 4.284.478

Apresentação do Projeto:

Este parecer está sendo emitido para análise da resposta à pendências, emitida pela nova proponente, ao parecer consubstanciado do CEP n. 4.209.334.

"A resposta de citocinas e sua participação no switch de subclasses de anticorpos ainda são pouco conhecidas na COVID-19, bem como o papel destes anticorpos no reconhecimento viral e potencial aplicação na prática clínica. Diante disso, este estudo se propõe a identificar o perfil de células e citocinas envolvido na indução da resposta imune humoral e celular, além do reconhecimento de proteínas do SARS-CoV-2 pelas diferentes subclasses de anticorpos encontradas no soro de indivíduos convalescentes da COVID-19 que apresentaram diferentes manifestações clínicas. Por fim, será avaliado o potencial uso desse perfil de citocinas/anticorpos como ferramenta para indicação do prognóstico, permitindo identificar precocemente casos prováveis de agravamento ou risco de evolução com complicações."

Objetivo da Pesquisa:

Objetivo Primário: O objetivo central da presente proposta e elucidar a cinética da resposta imune humoral e celular na COVID-19, explorando o perfil de citocinas
envolvido no switch das diferentes subclasses de anticorpos, bem como seu reconhecimento de

Apêndice A: Artigos produzidos como primeiro autor durante o período do doutorado e que não entraram no corpo da tese.

Bonyek-silva I, Nunes S, Bastos R, Lima R, Barbosa L, Grimaldi G, Soares MBP, Veras PST, Menezes J De, Brodskyn C, Tavares N. Obtainment of Macrophages from Human Monocytes to Assess Leishmania braziliensis Infection Rate and Innate Host Immune Response. Jove. doi:10.3791/62555, 2021.

Bonyek-Silva I, Nunes S, Santos RLS, Lima FR, Lago A, Silva J, Carvalho LP, Arruda SM, Serezani HC, Carvalho EM, Brodskyn CI, Tavares N. Unbalanced Production of LTB 4/PGE 2 Driven by Diabetes Increases Susceptibility to Cutaneous Leishmaniasis. Emerg Microbes Infect. doi:10.2139/ssrn.3529433, 2020.

Obtainment of Macrophages from Human Monocytes to Assess *Leishmania braziliensis* Infection Rate and Innate Host Immune Response

Icaro Bonyek-Silva[1,2], **Sara Nunes**[1,2], **Rana Bastos**[1], **Reinan Lima**[1,2], **Leilane Barbosa**[1,2], **Gabriela Grimaldi**[1], **Vinicius Rocha**[4], **Milena B. P. Soares**[1,4], **Patrícia S T Veras**[1,3], **Juliana de Menezes**[1,2], **Cláudia Brodskyn**[1,2,3], **Natalia Tavares**[1,2]

[1] Oswaldo Cruz Foundation, Gonçalo Moniz Institute [2] Federal University of Bahia [3] National Institute of Science and Technology (INCT), Research Institute in Immunology [4] Senai Institute for Innovation in Advanced Health Systems, National Service for Industrial Learning, Integrated Manufacturing and Technology Campus, SENAI - CIMATEC Salvador

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Abstract

Macrophages are multifunctional cells essential to the immune system function, and the primary host cell in *Leishmania braziliensis* (Lb) infection. These cells are specialized in microorganism recognition and phagocytosis, but also activate other immune cells and present antigens, as well as promote inflammation and tissue repair. Here, we describe a protocol to obtain mononuclear cells from peripheral blood (PBMC) of healthy donors to separate monocytes that then differentiate into macrophages. These cells can then be infected *in vitro* at different Lb concentrations to evaluate the ability to control infection, as well as evaluate host cell immune response, which can be measured by several methods. PBMCs were first isolated by centrifuging with Ficoll-Hypaque gradient and then plated to allow monocytes to adhere to culture plates; non-adherent cells were removed by washing. Next, adherent cells were cultured with macrophage-colony stimulating factor (M-CSF) for 7 days to induce macrophage differentiation. We suggest plating 2 x 10^6 cells per well on 24-well plates in order to obtain 2 x 10^5 macrophages. Fully differentiated macrophages can then be infected with Lb for 4 or 24 hours. This protocol results in a significant percentage of infected cells, which can be assessed by optical or fluorescence microscopy. In addition to infection index, parasite load can be measured by counting the numbers of parasites inside each cell. Further molecular and functional assays can also be performed in culture supernatants or within the macrophages themselves, which allows this protocol to be applied in a variety of contexts and also adapted to other intracellular parasite species.

Introduction

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Unbalanced production of LTB_4/PGE_2 driven by diabetes increases susceptibility to cutaneous leishmaniasis

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ABSTRACT

Poorly controlled diabetes mellitus leads to several comorbidities, including susceptibility to infections. Hyperglycemia increases phagocyte responsiveness, however immune cells from people with diabetes show inadequate antimicrobial functions. We and others have shown that aberrant production of leukotriene B_4 (LTB_4) is detrimental to host defense in models of bacterial infection. Here, we will unveil the consequences of high glucose in the outcome of *Leishmania braziliensis* skin infection in people with diabetes and determine the role of LTB_4 in human phagocytes. We show that diabetes leads to higher systemic levels of LTB_4, IL-6 and TNF-α in cutaneous leishmaniasis. Only LTB_4 correlated with blood glucose levels and healing time in diabetes comorbidity. Skin lesions of people with leishmaniasis and diabetes exhibit increased neutrophil and amastigote numbers. Monocyte-derived macrophages from these individuals showed higher *L. braziliensis* loads, reduced production of Reactive Oxygen Species and unbalanced LTB_4/PGE_2 ratio. Our data reveal a systemic inflammation driven by diabetes comorbidity in opposition to a local reduced capacity to resolve *L. braziliensis* infection and a worse disease outcome.

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KEYWORDS Diabetes; human leishmaniasis; *Leishmania braziliensis*; lipid mediators; LTB_4; PGE_2

Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia, which occurs when insulin is not produced (Type 1) or inefficiently recognized (Type 2) [1,2]. Both types of diabetes are associated with secondary complications, such as cardiovascular diseases, retinopathy, nephropathy, neuropathy, reduced wound healing and increased susceptibility to infections, particularly in the skin [1–7]. The recent increase in the burden of diabetes [8,9] and reports of abnormal cases of cutaneous leishmaniasis (CL) in individuals with diabetes [10,11] led us to investigate the consequences of association of these diseases. The main goal of this study is to evaluate the influence of diabetes and its inflammatory mediators in the outcome of *Leishmania braziliensis* skin infection.

Hyperglycemia is thought to cause a state called sterile inflammation, characterized by a low-grade inflammatory response [12]. Although inflammation is crucial to clear pathogens and to induce tissue repair, chronic and sustained inflammatory responses cause tissue damage, leading to immunopathology and worse disease outcome. The tissue damage results from increased production of TNF-α, Interleukin-1β, metalloproteases and recently, we have shown that lipid mediators, such as leukotrienes (LTB_4), when produced in abundance, increases susceptibility to skin infection in experimental models of diabetes [13–15]. These inflammatory mediators are also involved in CL caused by *Leishmania braziliensis,* the causative agent of CL in Brazil, resulting in skin ulcerative lesions [16]. Histological analysis of CL ulcers revealed rare presence of parasites and an intense inflammatory infiltrate, which can lead to a delayed healing process and chronic lesions [17]. In this context, we and others also have shown the participation of lipid inflammatory mediators, including LTB_4 and Prostaglandin E_2 (PGE_2) in phagocyte antimicrobial effector functions upon infection of different pathogens, including *Leishmania* spp. [18–21].

Lipid mediators are produced from the metabolism of arachidonic acid (AA) present in the cell membrane

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Apêndice B - Artigos produzidos em colaboração durante o período do doutorado e que não entraram no corpo da tese.

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Article

Keratinocytes and Activation of TREM-1 Pathway in Cutaneous Leishmaniasis Lesions

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Abstract: Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) amplifies the immune response, operating synergistically with Toll-Like Receptors (TLRs) in the production of inflammatory mediators. TREM-1 signaling depends on the adapter protein DAP12, which results in the activation of NFkB, the expression of inflammatory genes, and the release of antimicrobial peptides, such as Beta-defensin 2. We evaluated the activation of the TREM-1 signaling pathways in Cutaneous Leishmaniasis (CL) caused by Leishmania braziliensis and linage human keratinocytes exposed to these parasites since the host immune response against Leishmania plays a critical role in promoting parasite killing but also participates in inflammation and tissue damage. We analyzed publicly available transcriptome data from the lesions of CL patients. In the CL biopsies, we found increased expression of the molecules involved in the TREM-1 pathway. We then validated these findings with RT-qPCR and immunohistochemistry in newly obtained biopsies. Surprisingly, we found a strong labeling of TREM-1 in keratinocytes, prompting the hypothesis that increased TREM-1 activation may be the result of tissue damage. However, increased TREM-1 expression was only seen in human lineage keratinocytes following parasite stimulation. Moreover, no up-regulation of TREM-1 expression was observed in the skin lesions caused by other non-infectious inflammatory diseases. Together, these findings indicate that *L. braziliensis* (Lb) induces the expression of the TREM-1 receptor in tissue keratinocytes regardless of tissue damage, suggesting that non-immune skin cells may play a role in the inflammatory response of CL.

Keywords: *Leishmania*; inflammation; TREM-1; keratinocytes

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1. Introduction

Leishmaniasis is a complex of neglected tropical diseases caused by a protozoan parasite of the genus *Leishmania*. Among them, cutaneous leishmaniasis (CL) is the most frequent form of the disease. The immune response in cutaneous leishmaniasis caused by *Leishmania braziliensis (Lb)* is considered highly inflammatory, which is crucial for parasite killing but leads to tissue damage [1–4]. CL lesions are chronic skin ulcers with raised edges and a necrotic center. Its development depends on the *Leishmania* species in addition to a combination of factors associated with the host immune response, which defines different clinical outcomes. Several studies have suggested that the severity of CL is more associated with an exacerbated inflammatory response than a consequence of parasite burden. Chronic and exacerbated inflammation of CL has been associated with the high

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Citral modulates virulence factors in methicillin-resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for high morbidity and mortality rates. Citral has been studied in the pharmaceutical industry and has shown antimicrobial activity. This study aimed to analyze the antimicrobial activity of citral in inhibiting biofilm formation and modulating virulence genes, with the ultimate goal of finding a strategy for treating infections caused by MRSA strains. Citral showed antimicrobial activity against MRSA isolates with minimum inhibitory concentration (MIC) values between 5 mg/mL (0.5%) and 40 mg/mL (4%), and minimum bactericidal concentration (MBC) values between 10 mg/mL (1%) and 40 mg/mL (4%). The sub-inhibitory dose was 2.5 mg/mL (0.25%). Citral, in an antibiogram, modulated synergistically, antagonistically, or indifferent to the different antibiotics tested. Prior to evaluating the antibiofilm effects of citral, we classified the bacteria according to their biofilm production capacity. Citral showed greater efficacy in the initial stage, and there was a significant reduction in biofilm formation compared to the mature biofilm. qPCR was used to assess the modulation of virulence factor genes, and *ica*A underexpression was observed in isolates 20 and 48. For *ica*D, *seg*, and *sei*, an increase was observed in the expression of ATCC 33,591. No significant differences were found for *eta* and *etb*. Citral could be used as a supplement to conventional antibiotics for MRSA infections.

Staphylococcus aureus is an important and potentially lethal opportunistic pathogen. These bacteria have high virulence and the ability to acquire resistance mechanisms and pathogenic characteristics. *S. aureus* is normally associated with various infections acquired in the community and in hospitals[1–3].

Widespread and indiscriminate use of antibiotics can lead to the selection and antimicrobial resistance of bacterial isolates. The detection of antibiotic-resistant pathogens is relevant for therapeutic purposes, as well as to prevent the spread of resistant strains[4]. The *S. aureus* strain resistant to almost all β-lactam antibiotics, determined by a chromosomal gene *mecA* that encodes altered PBPs (PPB2a or PBP2'), is referred to as methicillin-resistant *Staphylococcus aureus* (MRSA) and can be found in hospital settings (HA-MRSA), as well as in the community (CA-MRSA)[5]. Infections caused by these bacteria are linked to higher mortality rates and higher treatment costs for an overburdened health system compared to infections caused by strains of *S. aureus* sensitive to methicillin (MSSA)[6–8].

The virulence potential of different isolates of *S. aureus* is determined by the presence or absence of virulence genes that encode staphylococcal enterotoxins (*ses*), Panton-Valentine leukocidin (*pvl*), exfoliatins (*eta* and *etb*),

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Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, under project PRJCA002557. The accession number is HRA000145. Further information about sequencing data can be found at https://bigd.big.ac.cn/gsa-human/browse/HRA000145.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
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Inflammasome Activation by CD8+ T Cells from Patients with Cutaneous Leishmaniasis Caused by *Leishmania braziliensis* in the Immunopathogenesis of the Disease

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TO THE EDITOR
Cutaneous leishmaniasis (CL) is characterized by an inflammatory response mainly mediated by CD4+ T cells producing IFN-γ, which are responsible for macrophage activation and intracellular Leishmania *braziliensis* parasite killing. We recently showed the importance of CD8+ T cells in the pathogenesis of human CL as skin lesions from patients with CL present higher frequencies of CD8+ T cells, contributing to the inflammatory response (Cardoso et al., 2015; Santos et al., 2013). Moreover, lesion size was found to positively correlate with the frequency of CD8+ T cells–expressing granzyme B. The cytotoxic response mediated by CD8+ T cells was not found to be linked to decreased parasite load in human macrophages infected in vitro (Cardoso et al., 2015; Santos et al., 2013).

Abbreviation: CL, cutaneous leishmaniasis

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RESEARCH ARTICLE Open Access

Multilocus sequence typing characterizes diversity of *Ureaplasma diversum* strains, and intra-species variability induces different immune response profiles

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Abstract

Background: *Ureaplasma diversum* is a pathogen found in the genital tract of cattle and associated with genital disorders such as infertility, placentitis, abortion, birth of weak calves, low sperm motility, seminal vesiculitis and epididymitis. There are few studies evaluating the genetic diversity of *U. diversum* strains and their influence on the immune response in cattle. Therefore, to better understand genetic relationships of the pathogenicity of *U. diversum*, a multilocus sequence typing (MLST) scheme was performed to characterize the ATCC 49782 strain and another 40 isolates recovered from different Brazilian states.

Results: Primers were designed for housekeeping genes *ftsH, polC, rpL22, rpoB, valS* and *ureA* and for virulence genes, phospholipase D (*pld*), triacylglycerol lipase (*tgl*), hemolysin (*hlyA*), MIB-MIP system (*mib,mip*), MBA (*mba*), VsA (*VsA*) and ribose transporter (*tABC*). PCRs were performed and the targeted gene products were purified and sequenced. Sequence types (STs), and clonal complexes (CCs) were assigned and the phylogenetic relationship was also evaluated. Thus, a total of 19 STs and 4 CCs were studied. Following the molecular analysis, six isolates of *U. diversum* were selected, inoculated into bovine monocyte/macrophage culture and evaluated for gene expression of the cytokines TNF-α, IL-1, IL-6, IL-10 and IL-17. Differences were detected in the induction of cytokines, especially between isolates 198 and BA78, promoted inflammatory and anti-inflammatory profiles, respectively, and they also differed in virulence factors.

Conclusion: It was observed that intra-species variability between isolates of *U. diversum* can induce variations of virulent determinants and, consequently, modulate the expression of the triggered immune response.

Keywords: *Mollicutes*, Genetic diversity, Sequence type, Clonal complex, Gene expression, Cytokines

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Original article

Ovarian hormones influence immune response to *Staphylococcus aureus* infection

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ABSTRACT

Objective: *Staphylococcus aureus* infections remain associated with considerable morbidity and mortality in both hospitals and the community. There is little information regarding the role of ovarian hormones in infections caused by *S. aureus*. The aim of this study was to evaluate the effects of ovariectomy in the immune response induced by *S. aureus*.

Methods: Female mice BALB/c were ovariectomized (OVX) to significantly reduce the level of ovarian hormones. We also used sham-operated animals. The mice were inoculated intraperitoneally with *S. aureus*. Blood samples were collected for leukocyte count and bacterial quantification. The uterus and spleen were removed and weighed to calculate the uterine and splenic indexes. Lungs were removed and fractionated for immunohistochemical analysis for macrophage detection (anti-CD68) and relative gene expression of IL-6, IL-1β and TNF-α by RT-PCR.

Results: Ovariectomy enlarged spleen size and generally increased circulating lymphocytes. OVX females experienced a continuation of the initial reduction of lymphocytes and a monocyte and neutrophil late response compared to shams ($p \geq 0.05$). Moreover, OVX females showed neutropenia after 168 h of infection ($p \geq 0.05$). Macrophage response in the lungs were less pronounced in OVX females in the initial hours of infection ($p \geq 0.01$). OVX females showed a higher relative gene expression of IL-1β, IL-6 and TNF-α in the lung at the beginning of the infection compared to sham females ($p \geq 0.01$). Among the uninfected females, the OVX control females showed a higher expression of IL-6 in the lung compared

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Metformin promotes susceptibility to experimental *Leishmania braziliensis* infection

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BACKGROUND Metformin (MET) is a hypoglycemic drug used for the treatment of diabetes, despite interference in host immunity against microorganisms. Cutaneous infection caused by pathogens such as *Leishmania braziliensis* (*Lb*), the agent responsible for cutaneous leishmaniasis (CL) in Brazil, represents an interesting model in which to evaluate the effects associated with MET.

OBJECTIVE To evaluate the modulatory effect of MET in *Lb* infection.

MATERIAL AND METHODS Experimental study of *Lb* infection and MET treatment in BALB/c mice and Raw 264.7 macrophages.

FINDINGS MET treatment interfered with lesion kinetics, increased parasite load and reduced macrophage proliferation. Low concentrations of MET in *Lb* culture allow for the maintenance of stationary parasite growth phase. *Lb*-infected cells treated with MET exhibited increased parasite load. While both MET and *Lb* infection alone promoted the production of intracellular reactive oxygen species (ROS), reduced levels of ROS were seen in MET-treated *Lb*-infected macrophages.

MAIN CONCLUSION Experimental treatment with MET interfered with the kinetics of cutaneous ulceration, increased *Lb* parasite load, altered ROS production and modulated cellular proliferation. Our experimental results indicate that MET interfere with the evolution of CL.

Key words: metformin - cutaneous leishmaniasis - immunomodulation - susceptibility - infection

Cutaneous leishmaniasis (CL) is a clinical form of the anthropozoonotic disease complex caused by protozoa of the genus *Leishmania*.[1] In Brazil, CL is commonly caused by *Leishmania braziliensis* (*Lb*) and is clinically characterised by one or more oval-shaped ulcerative lesions, or rounded, granulomatous-bottomed ulcerations with well-defined raised borders.[2,3] The low parasitic burden found in lesions results from a Th1-mediated intense inflammatory response, in addition to cytokine production, such as IFN-γ, TNF, IL-12 and IL-2. Intracellular death in these parasites results from the microbicidal action of IFN-γ-stimulated macrophages. However, exacerbated inflammation provokes tissue damage and initiates a pathological process culminating in the formation of ulcers on the skin of the host.[3,4,5]

In Brazil, pentavalent antimonials (Sb^{+5}) are the first-line drugs for *Lb* infection treatment. Despite high efficacy, the numerous side effects, such as cardiac, renal and hepatic toxicity, induced by these drugs consequently restricts use to certain groups of patients.[2] In addition, recent studies have demonstrated increasing resistance to these antimonials in several Latin American countries, as well as in India.[6,7,8] In light of this scenario, several studies have investigated alternative treatment strategies, such as combined therapy involving Sb^{+5} and immunomodulators, such as pentoxifylline, an inhibitor of TNF production, which seemed to accelerate cure in patients.[1,9]

The immunomodulatory effects of antidiabetic drugs, such as metformin (MET), has been described in cancer and infectious diseases, including tuberculosis and pneumonia caused by *Legionella pneumophila*.[10,11] Antidiabetic drug-induced effects include 5' AMP-activated protein kinase (AMPK) activation, signal transducer and activator of transcription 3 (STAT3) inhibition, and the increased production of mitochondrial reactive oxygen species (ROS).[12,13] Although described in other contexts, little is known about the action of MET in parasitic infections, such as *Lb* Considering the high prevalence of CL in Brazil, together with this drug's ample availability and low cost, the present study aimed to analyse the effects of MET on the immune response against *Lb* in an experimental mouse model.

MATERIALS AND METHODS

Experimental model design - After receiving ethical approval by the local Institutional Review Board for Animal Experimentation (protocol 013/2017), 6-week-old male isogenic BALB/c mice were obtained from the

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Leukotriene B_4 licenses inflammasome activation to enhance skin host defense

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The initial production of inflammatory mediators dictates host defense as well as tissue injury. Inflammasome activation is a constituent of the inflammatory response by recognizing pathogen and host-derived products and eliciting the production of IL-1β and IL-18 in addition to inducing a type of inflammatory cell death termed "pyroptosis." Leukotriene B_4 (LTB_4) is a lipid mediator produced quickly (seconds to minutes) by phagocytes and induces chemotaxis, increases cytokine/chemokine production, and enhances antimicrobial effector functions. Whether LTB_4 directly activates the inflammasome remains to be determined. Our data show that endogenously produced LTB_4 is required for the expression of pro-IL-1β and enhances inflammasome assembly in vivo and in vitro. Furthermore, LTB_4-mediated Bruton's tyrosine kinase (BTK) activation is required for inflammasome assembly in vivo as well for IL-1β–enhanced skin host defense. Together, these data unveil a new role for LTB_4 in enhancing the expression and assembly of inflammasome components and suggest that while blocking LTB_4 actions could be a promising therapeutic strategy to prevent inflammasome-mediated diseases, exogenous LTB_4 can be used as an adjuvant to boost inflammasome-dependent host defense.

inflammasome | leukotriene | skin | innate immunity

Upon infection, a fast and highly synchronized inflammatory response is mounted to restrict microbial growth and eventually eliminate the pathogen. Therefore, studies focusing on early events could unmask new players involved in the generation and magnitude of host defense. Inflammasomes are multiprotein intracellular platforms that detect both pathogen and host-derived products and induce the inflammatory response. The proteins that form the inflammasome consist of upstream sensors that belong to the nucleotide-binding oligomerization domain-like receptor (NLR) family, the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), and the downstream effector caspase-1. There are different NLR proteins, including NLRP1, 2, 3, 6, 7, and NLRC4, and absent in melanoma 2 (AIM2) (1, 2). The different NLRs recognize both distinct and overlapping stressors to elicit the maturation and secretion of the inflammatory cytokines IL-1β and IL-18. Upon cell stimulation with either pathogen- or damage-associated molecular patterns (PAMPs and DAMPs, respectively), the inflammasome is activated in two sequential steps: 1) transcription of the long forms (procytokines) of IL-1β and IL-18; and 2) the assembly of the inflammasome complex ASC/NLRP and procaspase-1, followed by autocatalytic cleavage of caspase-1, and processing and secretion of mature IL-1β and IL-18, as well as the production of lipid mediators, such as prostaglandins and leukotriene B_4 (LTB_4) (3–5). The secretion of these cytokines is followed by a form of programmed cell death called pyroptosis that releases DAMPs and further amplifies the inflammatory response. Increased production of IL-1β and IL-18, along with DAMPs generation, have been heavily implicated in the pathogenesis of a myriad of inflammatory diseases and facilitates antimicrobial activities (1, 6).

Staphylococcus aureus skin infection is controlled by the synchronized actions of structural cells (keratinocytes) and skin phagocytes. Inflammasome-dependent IL-1β production is required for neutrophil recruitment, abscess formation, and bacterial clearance (7, 8). The mechanisms underlying inflammasome activation and IL-1β production during skin infection is not well understood.

LTB_4 is a bioactive lipid mediator that is quickly produced by phagocytes, such as macrophages and neutrophils. LTB_4 synthesis involves several rate-limiting steps that include activation of phospholipase A_2 (PLA_2) and arachidonic acid (AA) release

Significance

Production of IL-1β is an essential component of the inflammatory response and host defense. IL-1β secretion is dependent on the activation of an intracellular platform termed inflammasome. The initial inflammatory signals that drive inflammasome activation remains elusive. Here, we show that the bioactive lipid leukotriene B_4 enhances both transcriptional and posttranscriptional programs that activate the inflammasome in vivo and in vitro. We identified critical signaling programs required for inflammasome assembly, IL-1β secretion, and its consequences in skin host defense. Our data also suggest that the prevention of LTB_4 actions might be an important therapeutic strategy to prevent IL-1β–dependent inflammatory diseases by inhibiting both first and second signals necessary for inflammasome activation.

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REVIEW
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Molecular Aspects of Dendritic Cell Activation in Leishmaniasis: An Immunobiological View

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Dendritic cells (DC) are a diverse group of leukocytes responsible for bridging innate and adaptive immunity. Despite their functional versatility, DCs exist primarily in two basic functional states: immature and mature. A large body of evidence suggests that upon interactions with pathogens, DCs undergo intricate cellular processes that culminate in their activation, which is paramount to the orchestration of effective immune responses against *Leishmania* parasites. Herein we offer a concise review of the emerging hallmarks of DCs activation in leishmaniasis as well as a comprehensive discussion of the following underlying molecular events: DC-*Leishmania* interaction, antigen uptake, costimulatory molecule expression, parasite ability to affect DC migration, antigen presentation, metabolic reprogramming, and epigenetic alterations.

Keywords: dendritic cell activation, *leishmania*- dendritic cell interaction, parasite uptake, dendritic cells migration, metabolism of infection, epigenetic modifications

INTRODUCTION

Important Considerations in Leishmaniasis

Leishmaniasis comprises a collection of neglected protozoan infections caused by unicellular organisms belonging to the genus *Leishmania* spp. According to the current World Health Organization estimation, 12 million people are affected by leishmaniasis and 350 million are at risk of infection worldwide (1–3).

The pathology of this disease results in a wide spectrum of clinical manifestations not only associated with the biological aspects of *Leishmania* species and strains, but also with host immune responses. Interestingly, it has been recently suggested that the clinical progression of the disease is influenced by several other factors, ranging from the host's nutritional status to the presence of RNA viruses in the *Leishmania* species (4–7).

These manifestations are dichotomically divided into Visceral (VL) and Tegumentary Leishmaniasis (TL). The former is characterized by the dissemination of parasites to visceral organs, while the latter branch includes Localized Cutaneous Leishmaniasis (LCL), a frequent form of TL in which ulcerated skin lesions are common. It has been abundantly reported that a modest fraction of LCL cases can evolve into mucosal lesions, which is termed as Mucocutaneous Leishmaniasis (MCL). Additionally, TL can also present as a variety of clinical manifestations, such as Disseminated Cutaneous Leishmaniasis (DCL), which comprises multiple nodular ulcerated lesions, whereas Diffuse Leishmaniasis (DL) is characterized by scattered non-ulcerated lesions (5, 8, 9).

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Integrated Analysis Reveals That miR-193b, miR-671, and TREM-1 Correlate With a Good Response to Treatment of Human Localized Cutaneous Leishmaniasis Caused by *Leishmania braziliensis*

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Localized cutaneous leishmaniasis (LCL) is a chronic disease characterized by ulcerated skin lesion(s) and uncontrolled inflammation. The mechanisms underlying the pathogenesis of LCL are not completely understood, and little is known about posttranscriptional regulation during LCL. MicroRNAs (miRNAs) are non-coding small RNAs that regulate gene expression and can be implicated in the pathogenesis of LCL. We investigated the involvement of miRNAs and their targets genes in human LCL using publicly available transcriptome data sets followed by *ex vivo* validation. Initial analysis highlighted that miRNA expression is altered during LCL, as patients clustered separately from controls. Joint analysis identified eight high confidence miRNAs that had altered expression ($-1.5 \leq$ fold change ≥ 1.5; $p < 0.05$) between cutaneous ulcers and uninfected skin. We found that the expression of miR-193b and miR-671 are greatly associated with their target genes, CD40 and TNFR, indicating the important role of these miRNAs in the expression of genes related to the inflammatory response observed in LCL. In addition, network analysis revealed that miR-193b, miR-671, and *TREM1* correlate only in patients who show faster wound healing (up to 59 days) and not in patients who require longer cure times (more than 60 days). Given that these miRNAs are associated with control of inflammation and healing time, our findings reveal that they might influence the pathogenesis and prognosis of LCL.

Keywords: ***Leishmania braziliensis*, microRNA, skin, transcriptome, TREM-1, human leishmaniasis**

INTRODUCTION

Leishmaniasis is a group of chronic diseases caused by intracellular protozoan parasites from the *Leishmania* genus that is transmitted by infected sandflies bites (1). Localized cutaneous leishmaniasis (LCL) is the most frequent form of these diseases and is characterized by ulcerated skin lesion(s) that can take a long time to heal (2). Human LCL caused by *Leishmania braziliensis* is associated with a chronic inflammation that is critical for parasite clearance but also for tissue injury and disease